Optimizing Microbial Screenings Using Controlled-Release Systems

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I dedicate this work to my father Norbert Scheidle

(1952-2007)

Preface

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Huber R, Scheidle M, Dittrich B, Klee D, Büchs J. 2009.
Equalizing growth in high-throughput small scale cultivations via precultures operated in fed-batch mode. Biotechnology and Bioengineering 103(6):1095-1102.

Abstract

Microbial screening experiments are of utmost importance for developing biotechnological processes. The cultivation parameters for selecting the most suited microorganisms during these screening experiments should match the parameters for the subsequent production process as exact as possible. It is absolutely necessary, to apply comparable cultivation conditions in small-scale screenings and large-scale production processes, to ensure a meaningful analysis of the screening experiments as well as a successful scale-up of its results. In the presented work, different facets of screening processes were analyzed and solutions for their optimization regarding the aforementioned general principles were investigated.

One key factor to improve the comparability of screening and production-scale experiments is to establish an online monitoring of cultivation parameters not only in large- but also in small-scale cultivations. To enhance the online information obtained during screening and process development in shake flasks, the RAMOS device for measuring respiration activities in shake flaks and a fiber optical, online pH-measurement technique were successfully combined.

To further improve the comparability between the different scales and to enable a more reliable scale-up of experiments, the cultivation strategy (i.e. the progression of pH-value and substrate concentration during the cultivation) has to be comparable in small- and in large-scales. Thus, the in large-scale applied pH-control and fed-batch operational mode have to be adapted to small scale screening experiments.

A disc-shaped polymer-based controlled-release system for pH-control in shake flasks was developed and applied in this thesis. It consists of a polymer matrix in which sodium carbonate as pH-control reagent is encased. When applied in cultivation media, this system releases sodium carbonate in pre-defined kinetics. With this system, it was possible to substantially reduce the buffer concentrations in shake flask cultivations of *Escherichia coli*, while the pH-values remained in the physiological range of microbial growth.

An additional physiological effect of the pH-value is its influence on the growth behavior of the microorganisms and thereby especially on the duration of the lag-phase. Different lag times of the microorganisms considerably affect the outcome of screening processes. In this work it could be shown that the initial pH-value of the cultivation media has an enormous strain dependent effect on the lag time of *E. coli* cultures. For three *E. coli* strains a lower initial pH-value resulted in a shorter lag phase and one strain showed the opposite behavior. This parameter should be considered in the design of production processes as well as of screening experiments.

Another analyzed facet of the screening process was the microbial growth in precultures. It could be demonstrated, that differences in the inoculum from precultures in shaken bioreactors have a tremendous effect on the microbial growth and thus on rational design of screening processes. Therefore, a new technique applying fed-batch mode in high-throughput precultivations for equalizing the initial parameters of subsequent screening experiments was introduced. For fed-batch cultivation in shake flasks, glucose containing polymer-based controlled-release discs were applied. For high-throughput applications a new fed-batch microtiter plate, with immobilized polymer-based controlled-release systems at the bottom of each well were presented. The newly developed fed-batch precultivation method enables equalized growth of all screened strains and will generate, therefore, more relevant and reliable data in subsequent main screening experiments. The feasibility of the presented concept has been proven for cultivations of *E. coli* and *Hansenula polymorpha*.

All these results demonstrate the importance of choosing the correct cultivation parameters for a successful microbial screening. Especially the control of the pH-value and the controlled-release of substrate are important for several aspects of screening experiments. The systems and methodologies described in the current work significantly improve screening procedures and the meaningful analysis and scale-up of the obtained results.

Zusammenfassung

Mikrobielle Screeningexperimente sind von großer Bedeutung für die Entwicklung biotechnologischer Prozesse, u.a. zur Auswahl des geeignetsten Mikroorganismus. Die Kultivierungsbedingungen in solchen Versuchen sollten den Bedingungen eines nachfolgenden Produktionsprozesses so gut wie möglich entsprechen. Dabei ist es absolut notwendig, vergleichbare Kultivierungsbedingungen in Kleinkulturscreenings und im großen Produktionsmaßstab zu verwenden, um sowohl eine aussagekräftige Analyse der Screeningexperimente treffen zu können, als auch ein erfolgreiches Scale-up zu realisieren. In dieser Arbeit werden verschiedene Facetten von Screeningexperimenten analysiert und Lösungen für die Optimierung in Hinsicht auf die vorher genannten, generellen Prinzipien untersucht.

Ein zentraler Punkt zur Verbesserung der Vergleichbarkeit von Screeningexperimenten und Versuchen im Produktionsmaßstab ist, eine online Überwachung von Kultivierungsparametern sowohl im großen, als auch im kleinen Maßstab zu etablieren. Zur Weiterentwicklung der online im Schüttelkolben gemessenen Parameter während des Screenings und der Prozessentwicklung, wurden das „Respiration Activity Monitoring System“ (RAMOS) zur Messung von Atmungsaktivitäten und eine optische online pH-Messtechnik erfolgreich kombiniert.

Zur Verbesserung der Vergleichbarkeit der verschiedenen Maßstäbe und um zuverlässiges Scale-up zu ermöglichen, müssen die Kultivierungsstrategien (z.B. der Verlauf der pH-Werte und der Substratkonzentrationen während der Kultivierung) im kleinen und im großen Maßstab vergleichbar sein. Aus diesen Gründen müssen die im großen Maßstab verwendete pH-Regelung und die Fed-batch-Betriebsweise für Screeningexperimente etabliert werden.

Ein scheibenförmiges, polymerbasiertes Freisetzungssystem zur pH-Kontrolle in Schüttelkolben wurde in dieser Arbeit vorgestellt und verwendet. Es besteht aus einer Polymermatrix, in die Natriumcarbonat als pH-Stellmittel eingebettet wurde. Wird es im Kultivierungsmedium verwendet, setzt es das Natriumcarbonat in einer bestimmten Kinetik frei. Mit Hilfe dieses Freisetzungssystems war es möglich, die Pufferkonzentrationen in *Escherichia coli*

Schüttelkolbenkultivierungen drastisch zu reduzieren. Dabei konnten die pH-Werte in einem Bereich gehalten werden, welcher für die Mikroorganismen physiologisch verträglich ist.

Die pH-Werte in Kultivierungsmedien haben einen starken physiologischen Einfluss auf das mikrobielle Wachstum von Mikroorganismen, wobei insbesondere die Länge der Lag-Phase beeinflusst wird. Unterschiedliche Lagzeiten von Mikroorganismen haben wesentliche Auswirkungen auf die Ergebnisse von Screeningexperimenten. In dieser Arbeit wird demonstriert, dass Start-pH-Werte von Kultivierungsmedien enorme, stammspezifische Effekte auf die Lagzeit von *E. coli* Kulturen aufweisen. Für drei verschiedene *E. coli* Stämme konnte eine verkürzte Lagzeit bei niedrigeren Start-pH-Werten und für einen weiteren Stamm das gegenteilige Verhalten nachgewiesen werden. Dieser Parameter sollte daher bei der Auslegung von Produktionsprozessen, als auch von Screeningexperimenten berücksichtigt werde.

Ein anderer analysierter Aspekt von Screeningexperimenten war das mikrobielle Wachstum von Vorkulturen. Es konnte demonstriert werden, dass Unterschiede im Inoculum von Vorkulturen in geschüttelten Bioreaktoren einen sehr großen Einfluss auf das Wachstum von Mikroorganismen haben und damit das rationale Design von Screeningprozessen maßgeblich beeinflussen. Aus diesem Grund wurde eine neue Technik zur Hochdurchsatz fed-batch Kultivierung von Vorkulturen präsentiert, mit der die Startparameter anschließender Screeningexperimente entsprechend angepasst werden können. Für die fed-batch Kultivierung in Schüttelkolben wurden glukosehaltige, polymerbasierte Freisetzungsscheiben verwendet. Für Anwendungen im Hochdurchsatz wurde eine neue Fed-batch-Mirkrotiterplatte vorgestellt, die am Boden von jedem Well ein immobilisiertes, polymerbasiertes Freisetzungssystem enthält. Diese neu entwickelte Methode zur fed-batch Vorkultivierung erlaubt das Angleichen des Wachstums aller gescreenten Stämme und generiert dadurch zuverlässigere Daten im anschließenden Screeningexperiment. Die erfolgreiche Anwendung dieser neuen Methode für die Vorkultivierung von *E. coli* und *Hansenula polymorpha* wird in dieser Arbeit dargestellt.

Die vorgestellten Ergebnisse demonstrieren, wie wichtig eine sorgfältige Auswahl der Kultivierungsparameter für ein erfolgreiches mikrobielles Screening ist. Insbesondere die Kontrolle des pH-Wertes und die kontrollierte Freisetzung von Substraten sind relevant für verschiedene Aspekte von Screeningexperimenten. Die in dieser Arbeit entwickelten Systeme und Methoden verbessern signifikant das Screening von Mikroorganismen und ermöglichen ein sinnvolles Scale-up in den Produktionsmaßstab.

Contents

Abbreviations

RAMOS	Respiration Activity Monitoring System	
OTR	oxygen transfer rate	[mol/L/h]
CTR	carbon dioxide transfer rate	[mol/L/h]
RQ	respiratory quotient	[-]
t	time	[h]
d_0	orbital shaking diameter	[mm]
n	shaking frequency	[rpm]
T	temprature	[°C]
V_L	filling volume per flask or per well	[mL]
DOT	dissolved oxygen tension	[%]
pH	pH-value	[-]
pH_0	initial pH-value	[-]
OD_{600}	optical density at 600 nm	[-]
OD_{t0}	initial optical density at 600 nm	[-]
c_{HA}	acid concentration of buffer	[mol/L]
$c_{HA,0}$	initial acid concentration of buffer	[mol/L]
c_{A-}	base concentration of buffer	[mol/L]
$c_{A-,0}$	initial base concentration of buffer	[mol/L]
c_{H+}	concentration of free protons	[mol/L]
c_{HA+A-}	total concentration of buffer	[mol/L]
pK_A	acid dissociation constant	[-]
a.u.	arbitrary units	
Feedrate	feeding rate	[g/L/h]
K_d	decay term	[1/h]

K_S	half-saturation constant	[g/L]
rel_{act}	relative activity	[-]
S	substrate concentration	[g/L]
S_0	initial substrate concentration	[g/L]
t_{acc}	duration of acceleration phase	[h]
t_{lag}	duration of lag phase	[h]
X	biomass concentration	[g/L]
X_0	initial biomass concentration	[g/L]
$Y_{x/s}$	substrate yield coefficient	[g/g]
μ	specific growth rate	[1/h]
μ_{max}	maximal specific growth rate	[1/h]

Figures

1. Introduction

1.1 Screening processes

Biotechnological process development consists of the selection of optimal producing microorganisms and culture conditions to produce value-added product and the scale-up into large-scale processes (Fig 1-1). The first step is the primary screening of potential production microorganisms. Here, a large number of experiments have to be performed in parallel to identify a small number of production microorganism candidates. In the secondary screening, the previously selected microorganisms are analyzed in detail to finally choose the production strain. Furthermore, the cultivation parameters such as the media, optimal pH-values etc. are investigated. After a production strain is selected the next step is the process development in lab- and pilot-scale fermentations to determine the optimal process conditions for a successful production process. Finally, the process is scaled up to the large production-scale.

The selection of the optimal strain determines the effectiveness of the large-scale production process. After screening, the characteristics of the chosen strain limit the productivity of the process. If the wrong strain has been chosen, these limits can not be compensated even with an optimal process development. Therefore, the characterization and the layout of optimal screening conditions are crucial issues (Stöckmann et al. 2003b). Here, optimal screening conditions means: as close as possible to the final production process.

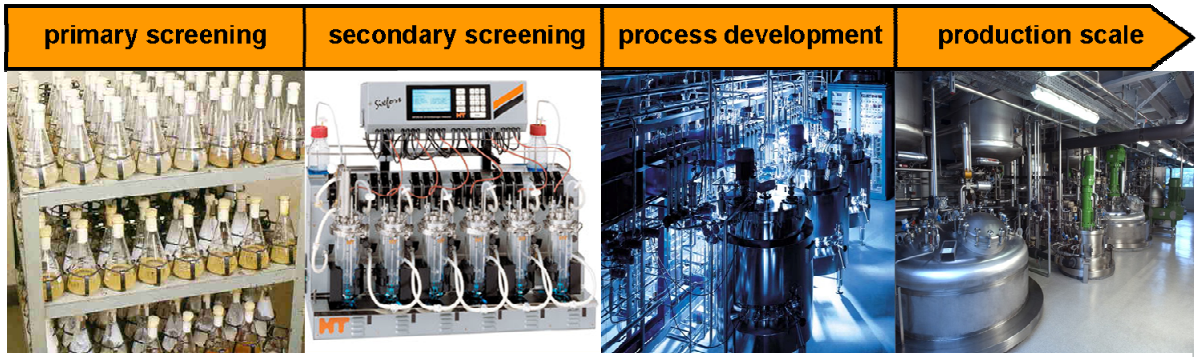

Fig. 1-1: Different scales in biotechnological process development. **Primary screening:** shake flasks on a shaker (Schott, Mainz, Germany); **secondary screening:** six parallel lab-scale fermentors (Infors, Einsbach, Germany) **process development:** three pilot-scale fermentors (Bioengineering, Wald, Switzerland), **production scale:** three production-scale fermentors (Lactosan, Kapfenberg, Austria) (adapted from (Jeude 2007))

1.2 Drawbacks of state-of-the-art screening processes

Freyer et al. defined a representative screening system with the following statement: "Optimization objectives perform similarly in screening and production system" (Freyer et al. 2004). Therefore, it is absolutely necessary to define these optimization objectives, such as the productivity, carbon yield or growth conditions, for the screening with the same values as in the production scale. To obtain similar and optimal results in the different scales it is essential to apply the cultivation parameters of the desired production processes also in the screening experiments. Only then, screening experiments can provide the microorganism optimally suited for the parameters applied in the subsequent large-scale process. Otherwise, microorganisms will be identified, that are optimally adapted to the parameters used in small-scale processes. Therefore, the problems and challenges of such screening processes have to be identified and solved, so that the small-scale optimally represents the production process and meets the requirements of high throughput applications.

Shaken bioreactors — especially shake flasks and microtiter plates — are typically applied in biotechnological high-throughput screening and process development (Büchs 2001; Duetz 2007; Rao et al. 2009). In shaken bioreactors batch operational mode, where all nutrients are provided from the beginning, is generally used for microbial cultivations (Kennedy et al. 1994; Kumar et al. 2004; Weuster-Botz et al. 2001). In the batch operational mode, high initial substrate concentrations and buffer concentrations to prevent excessive pH-drifts during the cultivation, are applied. In contrast, biotechnological processes in the industrial

scale are usually conducted in fed-batch cultivation mode, where substrates (i.e. the carbon source) are added during the cultivation and the pH-value is actively monitored and regulated (Kim et al. 2004; Larsson et al. 1997; Wittmann et al. 1995). These differences between the screening and the production process are tremendously influencing rational strain selection for an optimal production process and hinder an effective scale-up.

The high substrate and buffer concentrations applied in small-scale media may directly inhibit microorganism growth or product formation (Stöckmann et al. 2003b). For example, high substrate concentrations may repress product formation in catabolite repressed systems (Browning et al. 2005; Kramarenko et al. 2000; Stasyk et al. 2004; van Wijk 1968). Moreover, some microorganisms such as *Escherichia coli* and *Hansenula polymorpha* respond to high substrate concentrations by exhibiting overflow metabolism (Gellissen 2002; Xu et al. 1999), where large amounts of undesired by-products, such as acetic acid or ethanol, are secreted.

The different operational modes (batch or fed-batch) indeed change the metabolic parameters of microorganisms. Jeude et al. compared controlled-release fed-batch and batch mode fermentations of *H. polymorpha* (Jeude et al. 2006). They hereby found that delivering low levels of glucose avoids by-product formation caused by overflow metabolism. Moreover, derepression occurred for *H. polymorpha* RB11 pC10-FMD (P_{FMD}-GFP) leading to a 35-fold and 420-fold GFP formation on Syn6-MES and YNB mineral media, respectively, compared to batch mode. Oh et al. showed that also the carbon source significantly affects the transcription profile of this microorganism (Oh et al. 2004). They demonstrated that the expression of 32 genes changed with methanol as carbon source compared to glucose as carbon source (Oh et al. 2004).

The necessary high buffer and substrate concentrations in small-scale cultivation media lead to low water activity and high osmolarity. These conditions negatively influence the growth of microorganisms. For example, since the optimal osmolarity of a medium to cultivate *Escherichia coli* is approximately 0.3 Osmol/L, increasing or decreasing the osmolarity results in reduced bacterial growth rates (McLaggan et al. 1990; Record et al. 1998). Consequently, having to use high buffer and substrate concentrations might mask the screening for optimal production strains of microorganisms with low osmotolerance.

Therefore, finding a new system which would avoid the use of highly concentrated buffer would enhance the output of the screening.

Another important parameter for cultivating microorganisms is the pH-value. The best pH-range for cultivating *Escherichia coli*, i.e. 6.5-7.5, and varies with temperature (Davey 1994; Munro 1970). The metabolic activity of the cultivated microorganisms influences the pH-value of the surrounding medium in different ways. One of the most important pH affecting parameter in microbial cultivation processes is, for example, the consumption of ammonium, where the uptake of one ammonium molecule generates one proton (Christensen and Eriksen 2002; Siano 1995). Other examples are the the consumption of nitrogen containing complex compounds, the production and consumption of organic acids (i.e. acetic or lactic acid) and metabolically generated bicarbonate ions (Losen et al. 2004; Siano 1995). Therefore, the pH-values change significantly during fermentations. Consequently, a pH-control is absolutely vital to maintain physiological pH values during microbial cultivation (Weuster-Botz 2005).

An often underestimated problem while working with different clones in screening processes is the non parallel and non equal growth of batch cultures. These growth differences are caused by variances of individual clones regarding, for example, initial biomass concentrations, lag phases or specific growth rates. The non parallel growth in precultures can, therefore, have a tremendous effect on the performance of bioprocesses. Studier (2005) emphasizes that it is very difficult in high-throughput screening to obtain all of the cultures in a comparable state of growth. One strategy for achieving uniform conditions is to cultivate the microorganisms until the stationary growth phase (saturation) (Studier 2005). Though, when using this principle it has to be assured that the final pH of the cultures is not too acidic when they reach saturation. Moreover, the stationary growth phase in batch mode is characterized by tremendous structural and physiological effects on bacterial cells (Baev et al. 2006; Hengge-Aronis 1996; Huisman et al. 1996). Some authors have described that precultures remaining unequal time periods in the stationary phase show variations in the lag phase of main cultures (Hornbaek et al. 2004; Pin and Baranyi 2008). It is also known that the inoculum history is very important for the whole main cultivation process regarding reproducibility of growth kinetics (Ferenci 1999; Neves et al. 2001; Webb and Kamat 1993). Consequently, the growth of precultures to stationary phase can have negative effects on the following main cultivation. These factors make screening for the best producing clones very difficult with conventional approaches.

1.3 Screening tools

For optimizing screening processes different tools and devices were developed in the last years (Betts and Baganz 2006). Anderlei et al., for example, presented the Respiration Activity MOnitoring System (RAMOS) for online measurements of the respiration activity parameters (oxygen transfer rate (OTR), carbon dioxide transfer rate (CTR) and the respiratory quotient (RQ)) in shake flasks (Anderlei and Büchs 2001; Anderlei et al. 2004). Measuring OTR online during cultivation is the most suitable way to quantify the physiological state of aerobic microorganisms. For example, oxygen limitations, product inhibition and diauxic growth can be identified. The RAMOS device was successfully employed in different projects (Danielson et al. 2004; Hermann et al. 2001; Peter et al. 2004; Seletzky et al. 2006; Seletzky et al. 2007). Losen et al., for example, used it to optimize culture conditions and nutrient composition of an *Escherichia coli* fermentation medium in shake flasks (Losen et al. 2004). Furthermore, the RAMOS technology is especially suitable for optimizing screening cultures. Stoeckmann et al., for instance, demonstrated the impact of oxygen limitations during screening processes with *Hansenula polymorpha* (Stöckmann et al. 2003a; Stöckmann et al. 2003b) and Zimmermann et al. for *Corynebacterium glutamicum* (Zimmermann et al. 2006).

For the cultivation of microorganisms in microtiter plates (MTP) the BioLector was developed recently (Kensy et al. 2009; Samorski et al. 2005). The BioLector is able to measure online the microbial growth, fluorescence of reporter proteins, the pH and DOT in microtiter plates (MTPs) without interrupting the shaking of the plate. Therefore, the oxygen supply and the mixing of the cultures are permanently provided during the online measurements. This system consists of an orbital shaker, a MTP holder, an x-y positioning device, an optical fiber, a spectrophotometer for measuring the MTP and a computer. Furthermore, Huber et al. presented the Robo-Lector, a platform for high-throughput cultivations using a combination of the BioLector and a liquid handling robot (Huber et al. 2009). With these characteristics the BioLector is an excellent tool for screening experiments and process development.

1.4 Polymer-based controlled-release systems for fed-batch cultivation and pH-control in shaken bioreactors

To enhance the comparability of small-scale cultivations in shaken bioreactors and large scale fermentations some methods have been developed for cultivating microorganism in high-throughput, utilizing fed-batch mode in shake flasks or MTPs. Penula-Perälä et al. introduced a technique for cultivation in fed-batch mode in shaken bioreactors using an enzyme controlled glucose auto-delivering system (Panula-Perälä et al. 2008). Moreover, Jeude et al. presented an easy-to-use polymer-based controlled-release system for the fed-batch cultivation in shake flasks without the need for additional enzymes or equipment such as pumps and tubes etc. (Fig. 1-2) (Jeude et al. 2006). This controlled-release system contains a silicone elastomer (polydimethylsiloxane) matrix in which glucose is encased (Fig. 1-2a). In an aqueous system, such as culture media, the glucose is released in defined kinetics, depending on diffusion and swelling of the controlled-release system (Dittrich 2006; Jeude et al. 2006). Working principle is that water diffusing into the polymer matrix leads to the formation of micro-cracks in the polymer matrix. These micro-cracks then increase the release of the glucose from the matrix (Dittrich 2006).

A comparable technique for fed-batch cultivation in microtiter plates with an immobilized polymer-based controlled-release systems at the bottom of each well was published (Stöckmann et al. 2009) (compare chapter 6). With these controlled-release fed-batch MTPs it was demonstrated for *H. polymorpha* strains that fed-batch screening with glucose as carbon source is considerably better than screening in batch mode with glycerol or glucose as C-source (Scheidle et al. 2010; Stöckmann et al. 2009). In these studies, screenings of clone libraries of *H. polymorpha* were performed. Three different experimental set-ups were used to demonstrate the impact of the operational mode on the screening. The authors used batch cultivation with (1) glucose as substrate which catabolite represses the product formation and (2) glycerol as carbon source which is partially repressing, respectively. The third set-up used fed-batch cultivation with glucose (3) as limiting substrate using the controlled-release system. Interestingly, screenings in fed-batch mode with glucose as substrate resulted in different yeast strains being selected than those cultivated in batch mode with glycerol or glucose (Scheidle et al. 2010). The differences occurred due to the influence of the various cultivation parameters on the metabolism of the microorganisms. These results lead to the

conclusion that fed-batch screening is the preferable method for selecting production microorganisms.

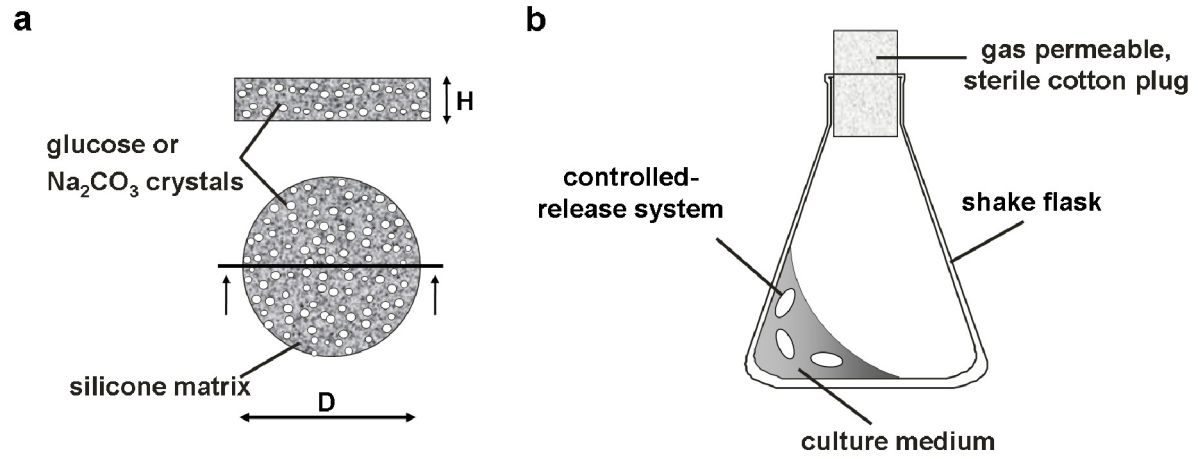

Fig. 1-2: Polymer-based controlled-release systems for shake flasks. a: Cut and top-view of a disc-shaped controlled-release systems with enclosed glucose or sodium carbonate crystals; b: regular shake flask with three disc-shaped controlled-release systems (adapted from Jeude et al. (2006))

One of the most important differences between small- and large-scale cultivations is controlling the pH of the medium. As discussed in chapter 1.2, high buffer concentrations are typically used to prevent excessive pH-drifts during microbial cultivations in shaken bioreactors. This high buffer concentration leads to high osmolarity and, therefore, significantly influences the cultivation process compared to large-scale fermentation. It is necessary to develop systems for pH-control in shaken bioreactors that do not need high buffer concentration in the medium. Weuster-Botz et al. presented a functional system for pH-control in shake flasks that applies pH-probes, pumps and other equipment (Weuster-Botz 2005; Weuster-Botz et al. 2001). However, because this system is very complex, it is impractical for high-throughput applications. Therefore, it is necessary to develop an easy-to-use system for pH-control in shake flasks without the need for additional equipment.

2. Objectives

In this work different crucial parts of microbial screening experiments were analyzed and methods were developed to optimize the screening process. Here, the main focus is to develop methods and cultivation conditions in small-scale that are comparable to large-scale processes. The main tools to realize this goal are controlled-release systems for fed-batch mode cultivation and for pH-control in small-scale cultivations.

To enhance the information output of screening processes and process development steps in small-scale cultivations in shake flaks, a combination of the RAMOS device and an optical fiber pH-measurement technique will be presented in chapter 3. With this combination an efficient development of controlled-release systems for pH-control in shake flasks will be possible.

One of the most important cultivation parameter is the pH-value. To control this value during fermentations in shaken bioreactors, high buffer concentrations are used. These highly concentrated buffers lead to high osmotic pressure (as described in chapter 1.2), which may negatively influence the growth of microorganisms. Additionally, strains screened in media with high osmotic pressure may possibly be selected for their osmotolerance but not for the parameters required in the large-scale process, such as the productivity. To reduce the buffer concentration in such media and to establish conditions in shake flasks that are comparable to actively pH-controlled large-scale fermentors, a polymer-based controlled-release system for pH-control in shake flasks will be presented in chapter 4.

Another important cultivation parameter in screening and production processes is the initial pH-value of the medium. As the initial pH-value dramatically influences the lag-phase of microorganisms, it is important to investigate this influence on the used microorganisms. In chapter 5 the influence of the initial pH-value on the lag-phase of four different *E. coli* strains is investigated.

Furthermore, different biomass concentrations and physiological states (i.e. growth phases) of various microorganisms in precultures have a tremendous influence on the main screening process. In this work, a new concept to cope with these problems in high-throughput precultures is elaborated. The strategy behind this concept is to use the fed-batch mode instead of the conventional batch mode in the precultivation step. For fed-batch cultivation, disc-shaped polymer-based controlled-release systems for shake flasks are used. Together with the Institute of Textile Chemistry and Macromolecular Chemistry (ITMC) of the RWTH Aachen University new MTPs with polymer-based controlled-release matrices immobilized at the bottom of each well are developed and applied in this work to demonstrate the high-throughput application of this strategy. The principle of this precultivation strategy is described by a mathematical model and studied experimentally with an *E. coli* and a *Hansenula polymorpha* strain (see chapter 6).

3. Measuring pH and oxygen transfer rates using a combination of fiber optical technique and RAMOS

3.1 Introduction

Up to now shaking bioreactors are the most commonly used reaction vessels in microbiology and biotechnology (Büchs 2001). Several thousand shake flask experiments are carried out annually for strain development, screening processes and media optimization in large companies (Büchs 2004; Freyer et al. 2004). For such applications, especially for media optimization, monitoring of cultivation parameters is essential. The information obtained from these experiments with online monitoring gives a better insight into limitations, inhibitions and the physiological state of the microorganisms during the cultivation, thus, allowing the development of optimized production processes in the biotechnology industry.

In chapter 1.3 the RAMOS device for online measurements of the respiration activity parameters (OTR, CTR and RQ) in shaking flasks is described. This system gives valuable information about, for example, oxygen limitations, product inhibition and diauxic growth of the cultivated and screened microorganisms. Other critical parameters during fermentation processes are pH-values and pH changes. There are different factors that affect the pH-value during the growth of microorganisms as discussed in chapter 1.2.

For monitoring pH-values of dairy starter cultures in 96-well microtiter plates, John et al. presented an optical method based on two different fluorophores (John et al. 2003). One fluorophore is pH sensitive (indicator) and the other one is pH insensitive (reference). To determine the actual pH in the solution the fluorescence intensities of both fluorophores are measured and with the ratio of both values the pH is calculated (John et al. 2003).

Another method for the fiber optical pH-measurement in small scale fermentation processes is the dual lifetime referencing (DLR). This method was published by Huber et al. for optical measurement of seawater salinity (Huber et al. 2000). DLR is based on the measurement of fluorescence decay times of an indicator. The intensity of the excitation light is modulated at a specific frequency and the over-all phase shift of the light emitted by a pH-indicator and a reference fluorophore is evaluated. The company Presens (Precision Sensing GmbH, Regensburg, Germany) commercialized this technology in form of sensor spots, e.g. for the pH measurement in microtiter plates (Hydroplates). This technique was used by Puskeiler et al. for atline pH-determination in microtiter plates (Puskeiler et al. 2005). Additionally, Kensy et al. demonstrated the application of this technology for online monitoring of dissolved oxygen and pH in continuous shaken *E. coli* cultivations performed in 24-well microtiter plates (Kensy et al. 2005).

In this chapter a fiber optical online pH-measurement, using DLR, is combined with the OTR-measurement in the RAMOS device is presented. This combination gives additional online information of cultures in screening and process development experiments. The successful combined application of both measurement techniques are demonstrated in *E. coli* cultivations.

3.2 Material and Methods

Organism and cultivation conditions

E. coli BL 21 pRset eYFP-IL6 was maintained at -80°C in LB medium with 100µg/mL ampicilin. This strain was described by Samorski et al. (2005) with an additional plasmid pLysS. Stock solutions contained 200 g/L glycerol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

All cultivations were performed at 37°C in 250 mL normal shaking flasks or RAMOS flasks, respectively, with 10 mL filling volume (V_L). Shaking machines (LS-W in case of RAMOS device; ISF-4-W in case of normal shaking flasks) with a shaking diameter (d_0) of 50 mm from Adolf Kühner AG, Birsfelden, Switzerland were used. The shaking frequency (n) was 350 rpm.

Media and solutions

A modified Wilms & Reuss (Jeude 2007; Wilms et al. 2001) medium was used for the cultivations (henceforth referred to as Wilms-MOPS medium). The medium consists of 20 g/L glucose; 5 g/L $(NH_4)_2SO_4$; 0.5 g/L NH_4Cl; 3 g/L K_2HPO_4; 2 g/L Na_2SO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 41,85 g/L 3-(N-morpholino)-propanesulfonic acid (MOPS); 0.1 g/L ampicillin; 0.01 g/L thiamine hydrochloride; 1 mL/L trace element solution (0.54 g/L $ZnSO_4 \cdot 7H_2O$; 0.48 g/L $CuSO_4 \cdot 5H_2O$; 0.3 g/L $MnSO_4 \cdot H_2O$; 0.54 g/L $CoCl_2 \cdot 6H_2O$; 41.76 g/L $FeCl_3 \cdot 6H_2O$; 1.98 g/L $CaCl_2 \cdot 2H_2O$; 33.39 g/L Na_2EDTA (Titriplex III)). The pH was adjusted to 7.3 with NaOH. All reagents were of analytical grade and purchased from Carl Roth GmbH & Co. KG, Karlsruhe, Germany.

Online measurement of oxygen transfer rates with the RAMOS device

The RAMOS device for online measuring of OTR in shake flasks was introduced by Anderlei et al. (Anderlei and Büchs 2001; Anderlei et al. 2004). The cultivations in the RAMOS device were performed in modified 250 mL Erlenmeyer flasks. In the RAMOS flasks the hydrodynamic conditions and the concentrations in the gas-phase of the head space are the same as in regular Erlenmeyer flasks with cotton plugs (Anderlei et al. 2004).

Samples and offline pH-measurement

Büchs 2001 described the problem of oxygen limitation while taking samples from the RAMOS flasks during the cultivation (Büchs 2001). Therefore, to measure offline data of the experiments, additional Erlenmeyer flasks were used for sampling. The *E. coli* were cultivated in parallel in these flasks under the same conditions as the cultivations in the RAMOS device. For the first sample 1 mL of the cultivation broth was withdrawn from the respective flask and the flask was refilled with sterile, purified water. Then, the same respective flask was used for a second sample. Here, only the flask taken for the first sample undergoes a short period of oxygen limitation.

The offline pH was measured with a CyberScan pH 510 (Eutech, Nijkerk, The Netherlands) pH meter at 37°C.

Online pH-measurement by fiber optical technique

Commercially available sterile pH sensitive sensor spots (Presens, Regensburg, Germany) were applied for the fiber optical online pH-measurement in the RAMOS flask. For gluing senor spots into the RAMOS flasks, silicone rubber compound (RS, Mörfelden-Walldorf, Germany) was used. The spot was glued with a wet stick under the clean bench at the inside glass wall of autoclaved flasks at the point with the highest flask diameter (Fig. 3-1). A pH-1 mini (Presens, Regensburg, Germany) with an optical fiber to illuminate and collect the emitted fluorescence from the sensor spot, was applied as pH meter.

The calibration was performed unsterile prior to a cultivation experiment with one sensor spot. For calibration six different buffers adjusted to different pH values between pH 4 and 9 were used to cover the measuring range. It was proven that the calibration was stable for the following online measurements with further sterile sensor spots from the same batch (data not shown). After 5 experiments a new calibration was performed.

For the online analysis of the optical pH measurement and the calculation of the pH-values from the phase shift values, a Visual Basic application in Microsoft Excel was kindly provided by Frank Kensy (m2p-laps, Aachen, Germany).

Combination of online pH-measurement and the RAMOS device

To fix the optical fiber on the RAMOS plate, a holder was mounted next to one of the RAMOS flasks (Fig. 3-1), so that the fluorescence intensities of the fluorophores in the sensor spot could be measured.

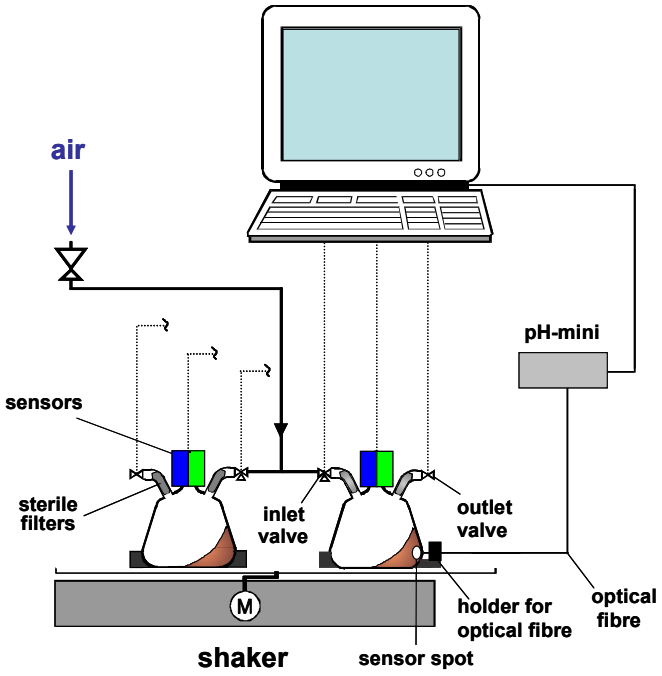

Fig. 3-1: Principal set-up of RAMOS in combination with fiber optical, online pH measurement.

3.3 Results and Discussion

Online measurement of OTR and pH in the Respiration Activity MOnitoring System (RAMOS)

E. coli BL 21 pRset eYFP-IL6 was cultivated in the RAMOS device, both with and without a sensor spot for pH online monitoring. Fig. 3-2 shows the development of the OTR and the pH in the RAMOS-flask with sensor spot.

The OTR of the cultivations with and without sensor spot proceeded more or less in parallel. In the flask with sensor spot the OTR curve is only slightly delayed. This might be due to slightly different inocula, for example, small variances in the initial biomass concentrations.

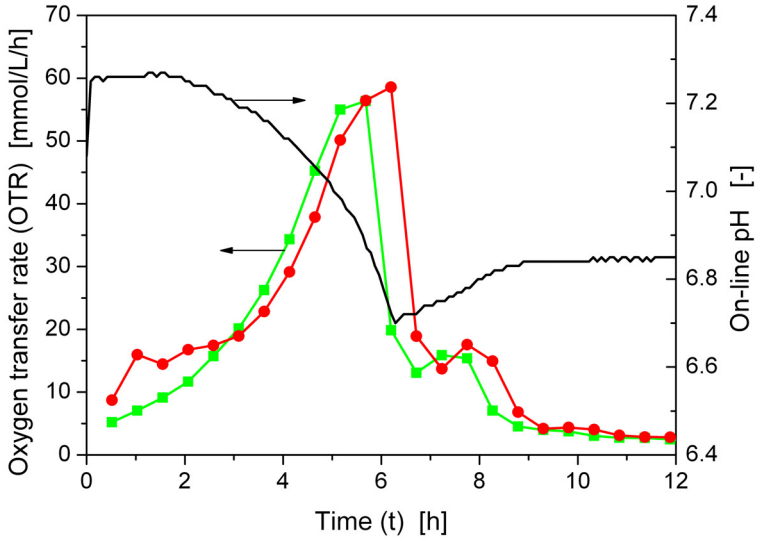

Fig. 3-2: Online measurement of OTR and pH in *E. coli* BL 21 pRset eYFP-IL6 cultivations; Wilms-MOPS medium; cultivation conditions: T = 37°C; d_0 = 50 mm; n = 350 rpm; V_L = 10 mL; OD_{t0} = 0.5; pH_0 = 7.3; legend: (■) OTR (flask without pH measurement); (●) OTR (flask with online pH measurement); (—) online pH

Without any lag-phase the bacteria started to grow so that the OTR increases directly. At the beginning of the cultivation the pH stays constant, because the acidification of the medium, caused by the metabolic activity of the microorganisms, is completely compensated by the buffer capacity. During the exponential growth of the microorganisms after 2 hours fermentation time, the pH decreases due to the increasing consumption of ammonium and the production of acetate in the overflow metabolism as discussed by Christensen et al. (Christensen and Eriksen 2002). After 6 h the first carbon source glucose is exhausted and therefore, the OTR plummeted. Thereafter, the OTR shows a second peak, while simultaneously the pH rises. From this point the microorganisms consume acetate as second carbon source. Due to the removal of acidic acetate, the pH of the medium increases until the acetate is depleted and the microorganisms enter the stationary growth phase after 9 h. In the stationary growth phase the pH stays constant and the OTR declines to a low level.

Comparison of offline and online pH-measurement

To compare online and offline pH measurements *E. coli* BL 21 pRset eYFP-IL6 was cultivated in the RAMOS device and in parallel in normal shake flasks for sampling. Fig.3-3 shows the courses of both measured pH values.

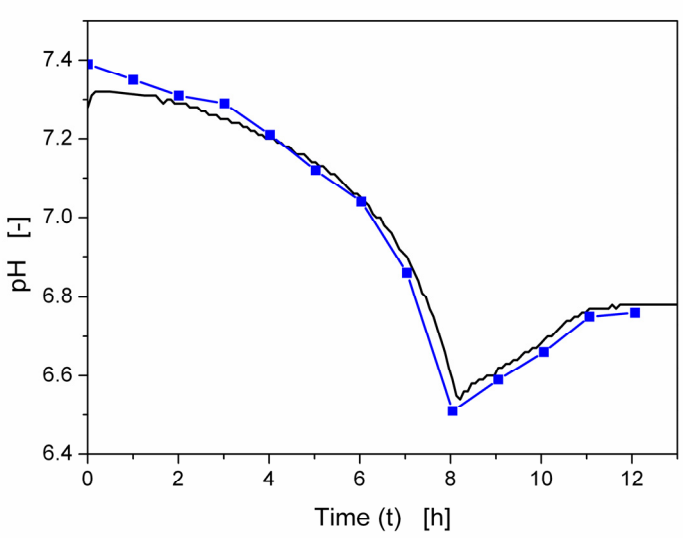

Fig. 3-3: Comparison of offline and online pH measurements in an *E. coli* BL 21 pRset eYFP-IL6 cultivation; Wilms-MOPS medium; cultivation conditions: T = 37°C; d_0 = 50 mm; n = 350 rpm; V_L = 10 mL; OD_0 = 0.5 pH_{t0} = 7.3; legend: (—) online pH; (■) offline pH

The pH courses show typical shapes for cultivations of *E. coli* BL 21 pRset eYFP-IL6 under the given conditions, which was already discussed in Fig. 3-2. Both curves proceed in parallel and the maximum difference between online and offline pH measurement was ± 0.05 pH. The highest difference occurs at the beginning of the cultivation.

The error in the different measurements averages to pH-values of ± 0.02, which lies in sum of the accuracies of the Eutech pH meter (Eutech Instruments Europe B.V., Nijkerk, Netherlands) and the pH-mini (Precision Sensing GmbH, Regensburg, Germany) with 0.01 pH-values, respectively (according to manufacturers). Therefore, the online pH measurement in the RAMOS device gives reliable results compared to the offline measurement.

3.4 Conclusion

The combination of the fiber optical, online pH and OTR measurements in the RAMOS device was successfully applied. The presented technique enables pH-measurements in RAMOS flasks without sampling and stopping the shaking machine. Therefore, mass transfer and mixing are not interrupted during the cultivation. Seletzky et al. showed that interruptions could lead to anaerobic periods during cultivation and changes in the metabolic activity of the microorganisms (Seletzky et al. 2006). Another advantage of this system is that the filling volume in the flasks does not change due to sampling, which allows an undisturbed growth of

microorganisms. Moreover, pH-values during cultivations in RAMOS flasks and normal shake flasks are comparable.

The pH effects on the OTR during growth of microorganisms, e.g. inhibited growth due to low-pH values, can easily be identified with this measuring setup. Furthermore, the online pH-measurement gives a higher resolution than the offline pH-measurement. For instance, pH changes could be resolved more precisely in the online measured pH-value compared to the offline measurement, thus, providing better information about the process.

Adaptation and regulation of growth conditions is of utmost importance for microorganisms with complex growth behavior, which for example produce and/or consume different pH affecting substances like acetate, lactate or glutamate. For instance, *Gluconobacter oxydans* produces 5-keto-D-gluconate, 2-ketogluconate and 2,5-diketogluconate on glucose as sole carbon source, whereas the product formation is highly dependent on the pH-value and the oxygen supply during the fermentation (De Ley et al. 1984; Levering et al. 1988; Qazi et al. 1991; Silberbach et al. 2003). Thereby the pH-profile of the *G. oxydans* cultivation must be adjusted to the desired product (Losen et al. 2004) and the acidification caused by the products must be considered. The here presented measurement technique is especially useful for media and cultivation optimizations and oxygen supply and additionally, buffer concentrations of media can be analyzed and adapted to the requirements of the microbial growth.

The combination of the online OTR and pH measurement gives a lot of information about the cultivation and, therefore, is a powerful tool for monitoring of shake flask experiments for screenings as well as for process development. This additional pH-monitoring reduces the gap between small-scale and large-scale information output of cultivations. Furthermore, the development of a controlled-release system for pH-control in shake flasks will be improved with the online pH-measurement (see chapter 4).

4. Controlling pH in shake flasks using polymer-based controlled-release systems with pre-determined release kinetics

4.1 Introduction

Biotechnological processes are usually conducted in fed-batch cultivation mode with active pH-monitoring and regulation. In contrast, shake flask experiments are usually conducted in batch mode without active pH-control, but with high initial buffer concentrations to prevent excessive pH-drifts during the cultivation (Jeude et al. 2006; Kumar et al. 2004) (see chapter 1.2). High buffer and substrate concentrations in small-scale cultivation media lead to low water activity and high osmolarity. These conditions may inhibit the growth of microorganisms. For example, since the optimal osmolarity of a medium to cultivate *Escherichia coli is* approximately 0.3 Osmol/L, increasing or decreasing the osmolarity here results in reduced bacterial growth rates (McLaggan et al. 1990; Record et al. 1998). Consequently, the application of high buffer and substrate concentrations in the screening for optimal production strains might handicap microorganisms with high potential but low osmotolerance. Therefore, a new system which would avoid the use of buffer in screening experiments would enhance the output of screening projects.

For fed-batch cultivation of microorganisms in shake flasks a polymer-based controlled-release system without the need of additional equipment or enzymes was developed by Jeude et al. (2006) (see chapter 1.4). This controlled-release of glucose in fed-batch cultivations in shake flasks thereby allows the user to reduce the initial substrate concentration in the medium.

The pH-value is an important parameter for cultivating microorganisms. The best pH-range for cultivating e.g. *Escherichia coli* is 6.5-7.5, and varies with temperature (Davey 1994; Munro 1970). The metabolic activity of the cultivated microorganisms influences the pH-value of the surrounding medium in different ways as described in chapter 1.2 and 3.

Therefore, the pH-value changes significantly during fermentations. Consequently, a pH-control is absolutely vital to maintain physiological pH values during microbial cultivation (Weuster-Botz 2005).

One of the most important differences between small- and large-scale is the control of the pH of the medium. Weuster-Botz et al. presented a system for pH-control in shake flasks that applies pH-probes, pumps, storage vessels for pH controlling agents and other equipment (Weuster-Botz 2005; Weuster-Botz et al. 2001). However, as this system is complex, it is impractical for high-throughput applications.

An easy-to-use polymer-based controlled-release system for keeping the pH in shake flasks in reasonable range, based on the fed-batch system described by Jeude et al. (Jeude et al. 2006) (see chapter 1.4), is presented in this chapter. This newly developed system consists of a biocompatible silicone matrix (polydimethylsiloxane) in which the alkaline reagent sodium carbonate is embedded (Fig. 1-2a). This sodium carbonate is then gradually released from the system in pre-determined kinetics and thus increases the pH-value of the medium. The focus of this chapter is to reduce buffer concentrations in cultivation media in order to decrease the osmolarity of small-scale media so that they can be better compared to large-scale media. Furthermore, the pH values during the fermentation should stay in the physiological range of the microorganisms. To demonstrate the applicability of this controlled-release system in shake flask cultures, *Escherichia coli* K12 and *Escherichia coli* BL21 pRSET eYFP-IL6 were used as model microorganisms and were cultivated in media containing glycerol or glucose as carbon source, respectively.

4.2 Material and Methods

Organisms

E. coli K12 and *E. coli* BL21 pRSET eYFP-IL6 (Samorski et al. 2005) were used as model microorganisms. Stock solutions were maintained in glycerol at -80°C in LB medium. *E. coli* BL21 pRSET eYFP-IL6 cultures additionally contained 100 µg/mL ampicilin.

Media and Solutions

Modified Wilms & Reuss synthetic medium (henceforth referred to as Wilms-MOPS medium) was used for *E. coli* cultivations (Jeude 2007; Wilms et al. 2001). The medium

consists of 20 g/L glucose or 20 g/L glycerol; 5 g/L $(NH_4)_2SO_4$; 0.5 g/L NH_4Cl; 3 g/L K_2HPO_4; 2 g/L Na_2SO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 41.85 g/L (0.2 M) 3-(N-morpholino)-propanesulfonic acid (MOPS); 0.1 g/L ampicillin; 0.01 g/L thiamine hydrochloride; 1 mL/L trace element solution (0.54 g/L $ZnSO_4 \cdot 7H_2O$; 0.48 g/L $CuSO_4 \cdot 5H_2O$; 0.3 g/L $MnSO_4 \cdot H_2O$; 0.54 g/L $CoCl_2 \cdot 6H_2O$; 41.76 g/L $FeCl_3 \cdot 6H_2O$; 1.98 g/L $CaCl_2 \cdot 2H_2O$; 33.39 g/L Na_2EDTA (Titriplex III). The pH was adjusted to 7.5 with NaOH. The typical MOPS buffer concentration of 0.2 M was used for precultures and for reference cultivations with glucose and glycerol, respectively. For other experiments different MOPS buffer concentrations and different initial pH-values were used and are mentioned in the respective experiment description.

Cultivation

For online monitoring of oxygen transfer rates (OTR) of all cultures a Respiration Activity Monitoring System (RAMOS) device, fabricated in-house and previously described by Anderlei et al. (Anderlei and Büchs 2001; Anderlei et al. 2004), was used. A commercial version of this device is available from HiTec Zang GmbH (Herzogenrath, Germany) or Kühner AG (Birsfelden, Switzerland). The following cultivation parameters were applied: 350 rpm shaking frequency, 50 mm shaking diameter, 10 mL filling volume in 250 mL RAMOS flasks. Precultures and main cultures were cultivated in Wilms-MOPS synthetic medium. For inoculating main cultures, fresh precultures (grown to an OTR of ca. 0.05 mol/L/h) were centrifuged, washed in 5 mL fresh medium, centrifuged again and the pellet was finally resuspended in 5 mL medium. Then optical densities (OD) were measured and used for calculating the required inoculation volume for each experiment. The initial pH-values of the main cultures were set to 7 or 7.5 as indicated in the respective experiment descriptions.

The polymer-based controlled-release system containing Na_2CO_3

To keep the pH-value of a cultivation in a narrow range without using high buffer concentrations, a polymer-based controlled-release system with embedded Na_2CO_3 was developed. In this work disc-shaped controlled-release systems were used. The release system was composed of solvent-free two-component (called component A and B by the manufacturer) silicone Sylgard™184 as well as the catalyst Syl-off™ 4000 (Dow Corning, Wiesbaden, Germany) in a concentration of 0.1 % (w/w). The ratio between the two components A and B was 10:1 as recommended by the manufacturer. Analytical grade

Na_2CO_3 was supplied by Sigma Aldrich (Crailsheim, Germany). The Na_2CO_3 was milled with a vibration micromill (Spartan™, Fritsch, Idar-Oberstein, Germany) in a high-grade steel mortar and then sieved through test sieves (Fritsch, Idar-Oberstein, Germany). The fraction with particle sizes ranging from 20 to 50 µm was used. First, a mixture consisting of component A of Sylgard™184, Na_2CO_3 (30 % (w/w)) and catalyst was weighed and degassed in a desiccator in a 30 mbar vacuum for 0.5 h. Then, component B was added. The finished mixture was subsequently casted as a thin foil onto a glass plate with a casting knife (gap 1.1 mm) and then cross-linked at 50°C in a convection oven for 3 h. Then discs having a diameter of 15 mm were stamped out (surface area 405.26 mm^2) and applied for the experiments. The disc-shaped controlled-release systems then gradually release the embedded Na_2CO_3 in an aqueous system at a pre-determined rate, so that the medium was alkalized during cultivation, thus counteracting any biological acidification.

Measurement of Na_2CO_3-release kinetics from the polymer-based controlled-release system

For measuring the release kinetics of Na_2CO_3 from the controlled-release system, an Na^+-ion selective electrode was used. With this electrode it is generally possible to determine the concentration of Na^+-ions within the range from 10^{-6} mol/L to 1 mol/L. The electrode consists of two half cells, a reference electrode (inLab Reference Pro) and the electrode for the Na^+-ions. The measuring instrument (SevenMulti) and the electrodes were purchased from MettlerToledo (Gießen, Germany). All salts used were purchased in the highest purity from Sigma Aldrich (Munich, Germany). Destilled water was prepared in a Millipore unit (Millipore, Schwalbach, Gemany). It was used for the preparation of the solutions and kept at a temperature of 20°C. As bridge electrolyte inside the Na^+-ion selective electrode a 0.1 mol/L NH_4Cl-solution was applied. As ion adjustment buffer (ISA) an NH_4Cl/NH_3-solution was prepared by adding 200 g NH_4Cl to 50 ml concentrated NH_3-solution and the volume was filled up with water to a final value of 1 L. For the measurement, the destilled water with ISA-Buffer was adjusted to a pH-value of 7 with 1 M KOH. As master solution for the calibration and to store the Na^+-ion selective half cell, a 0.1 M NaCl solution was prepared. For the calibration sodium chloride was dried 2 h with 120 °C and 5.845 g were weighed and filled up with water to 1L.

For the calibration the comparable conditions such as in the biological experiments were applied (T = 37 °C; agitated with a magnet stirrer). The calibration was done at pH values

from 3 to 8 and with sodium carbonate concentrations in the applied concentration range in the experiments of $1*10^{-4}$; $0.5*10^{-4}$; $1*10^{-3}$; $0.5*10^{-3}$; $1*10^{-2}$; $0.5*10^{-2}$ and $1*10^{-1}$ mol/L. The measurement was accomplished with normal sensitivity and in the automatic measuring mode. For measuring the release from the controlled-release system, 70 ml ISA were employed for each system. After the experiment the measured controlled-release system was dried and reweighed. In this work disc-shaped controlled-release systems with a diameter (D) of 15 mm a height (H) of 1.1 mm with a sodium carbonate content of 30% (w/w) were tested.

Analytical methods

To measure off-line data of the experiments, additional Erlenmeyer flasks were used for sampling. The investigated *E. coli* strains were cultivated in parallel in these flasks under the same conditions as the cultivations in the RAMOS device. For the first sample, depending on the particular experiment, 1 mL or 2 mL of the cultivation broth was withdrawn from the respective flask and the flask was refilled with sterile, purified water. Then, the same respective flask was used for one additional second sample. Each flask was only applied for two samples.

Optical densities were measured at 600 nm (OD_{600}) with an Uvikon 922 spectrophotometer (Kontron, Milano, Italy) except for the experiment depicted in Fig. 4-3 a Thermo Scientific Genesys 20 spectrophotometer (Waltham, MA, USA) was used. The samples were diluted with fresh medium to measure the OD in the linear range of the photometer and were at least determined twice.

Off-line pH-values were measured with a CyberScan pH 510 (Eutech, Nijkerk, The Netherlands) and with a Titroline alpha (Schott Instruments, Mainz, Germany). For online measurement of the pH during cultivations, a combination of a RAMOS device and a (Presens, Regensburg, Germany), as described in chapter 3, was used. With the fiber optical pH-measuring technique the pH-value was measured every 5 min.

Glycerol, glucose and acetate concentrations were measured with a Dionex HPLC (Dionex, Sunnyvale, USA) with an Organic Acid-Resin 250 x 8mm (CS-Chromatographie, Langerwehe, Germany) and a Skodex RI-71 detector. Sulphuric acid in a concentration of 5 mM was used as solvent at a flow rate of 0.6 ml/min and a temperature of 60°C.

4.3 Results and Discussion

In Fig. 4-1 the sodium carbonate release kinetics of the controlled-release system with 30% (w/w) sodium carbonate content used in this work and, additionally, the pH-course in the applied buffer are illustrated. During the first 2 h a rapid release of the salt occurred and caused a fast increase in the pH-values. Then, the sodium carbonate is nearly linearly released. In this time the pH-values also increase to a value of about 8.4 after 24 h, due to the released sodium carbonate. After 24 h about 0.14 mg sodium carbonate were released per mg of used controlled-release system. The rapid release and pH increase at the beginning of the experiment had to be considered in all biological experiments, because of lag phases and initially slow growing microorganisms where only few protons are produced.

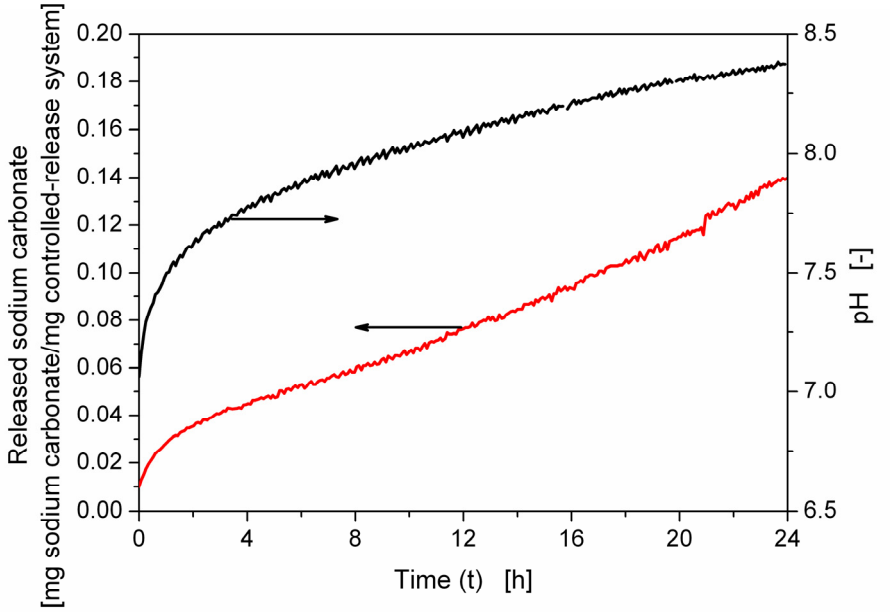

Fig. 4-1: Release kinetics and pH-course of one sodium carbonate containing (30% (w/w)) disc-shaped controlled release system. Released sodium carbonate (—); pH (—); doubly distilled water with ISA buffer; T = 37°C; pH_0 = 7; controlled-release system: D = 15 mm; H = 1.1 mm;

To investigate the influence of the buffer concentration and, therefore, of the osmotic pressure on the metabolic activity of *E coli* BL21 pRSET eYFP-IL6, cultivations with different MOPS buffer concentrations were conducted (Fig. 4-2).

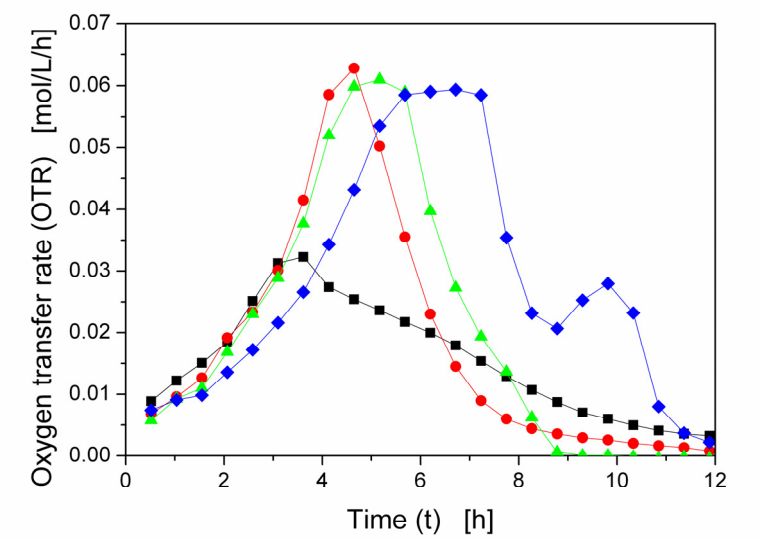

Fig. 4-2: Oxygen transfer rate (OTR) of *E. coli* BL21 pRSET eYFP-IL6 during growth in Wilms-MOPS medium with different buffer concentrations. With 0.2 M MOPS buffer (◆), with 0.1 M MOPS buffer (▲), with 0.05 M MOPS buffer (●), without MOPS buffer (■); experimental conditions: Wilms-MOPS medium with 20 g/L glucose, 37°C; 10 mL filling volume; shaking diameter (d_0) 50 mm; 350 rpm; $OD_{600,\alpha}$ = 0.5; pH_0 = 7.5

The reference cultivation with 0.2 M MOPS buffer depicted in Fig. 4-2, showed the typical metabolic activity of the microorganisms with the aforementioned cultivation parameters as described in Fig. 3. After about 5.5 h the OTR peaked at a value of ca. 0.06 mol/L/h and then formed a plateau, which indicates oxygen limitation (Anderlei and Büchs 2001). When the OTR plummeted after ca. 7 h the carbon source glucose was completely consumed. The second peak, with approximately 0.3 mol/L/h, indicates diauxic growth. Here, acetate is consumed, that was produced during the oxygen limitation.

The cultivation with 0.1 M MOPS buffer peaked about one hour earlier, than the culture with 0.2 M MOPS buffer concentration, after ca. 4.5 h. A short oxygen limitation can than be recognized before the OTR decreased slowly in a triangle-shaped form. This triangle-shaped form indicates acidic pH-values that impair the metabolic activity of the microorganisms (Anderlei and Büchs 2001).

The maximum specific growth rate (μ_{max}) increased between the cultivations with 0.2 M and 0.1 M MOPS buffer by ca. 20%, from 0.44 1/h to 0.53 1/h. This increased growth rate can be

attributed to the higher osmolarity in the medium with 0.2 M MOPS buffer. The Wilms-MOPS media with 0.2 M and 0.1 M MOPS buffer concentration have an osmolarity of ca. 0.65 Osmol/L and ca. 0.48 Osmol/L, respectively. Record et al. described a linear decrease of the specific growth with increasing osmolarities of the medium above 0.3 Osmol/L (Record et al. 1998), whereas the specific growth rate decreases in minimal medium by half from ca. 0.3 Osmol/L to 1 Osmol/L.

Application of 0.05 M MOPS demonstrates the same OTR curve and μ_{max} as the cultivation with 0.1 M MOPS buffer until 4.5 h. Here, the OTR decreased earlier in a triangle-shaped form. The low buffer concentration leads to a fast acidification of the medium and, therefore, to impaired metabolic activity.

The cultivation without any buffer shows the same metabolic activity and μ_{max} as the culture with 0.1 M MOPS buffer until after 3.5 h the OTR peaks at ca. 0.03 mol/L/h. After this peak the OTR slightly decreases. Without buffer, the culture acidifies even earlier and the growth is hampered due to suboptimal pH-values.

To demonstrate the application and the feasibility of the sodium carbonate containing controlled-release systems for controlling the pH in shake flasks, cultivations with *E coli* BL21 pRSET eYFP-IL6 in media with different MOPS buffer concentrations and, in addition, controlled-release systems were performed (Fig. 4-3).

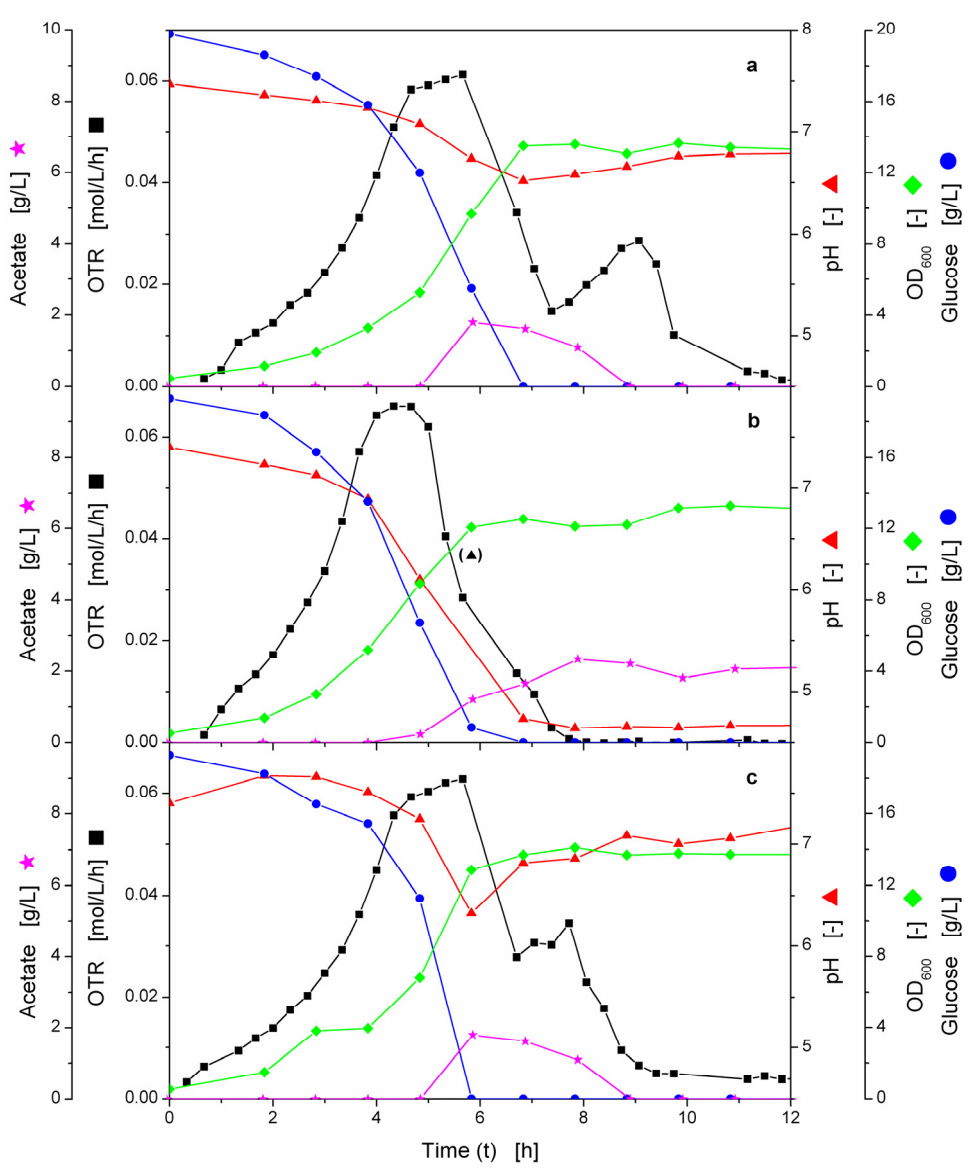

Fig. 4-3: Comparison of the oxygen transfer rate (OTR), pH and glucose concentration during the cultivation of *E. coli* BL21 pRSET eYFP-IL6 with different buffer concentrations and controlled-release systems. a: with 0.2 M MOPS buffer, b: with 0.1 M MOPS buffer, c: with 0.1 M MOPS buffer and additional 3 controlled release systems. OTR (■); pH (▲); glucose concentration (●); OD_{600} (◆); acetate concentration (★); experimental conditions: Wilms-MOPS medium with 20 g/L glucose, 37°C; 10 mL filling volume; shaking diameter (d_0) 50 mm; 350 rpm; $OD_{600,\alpha}$ = 0.5; pH_0 = 7.5; controlled-release systems: D = 15 mm; H = 1.1 mm; 30% (w/w) Na_2CO_3

In the reference cultivation with *E. coli*, depicted in Fig. 4-3a, a MOPS buffer in a concentration of 0.2 M was applied. This cultivation showed the same shape depicted in Fig. 4-2. During the oxygen limitation between 4.5 h and 6 h about 1.9 g/L acetate were produced. After 6 h cultivation time the glucose was depleted and the OTR plummeted. Then a second OTR maximum at ca. 0.03 mol/L/h is observed at about 9 h. From 7 to 11 hours of cultivation, the previously produced acetate was consumed as carbon source. During this time no biomass was produced. The pH-curve decreased during the exponential growth of the microorganisms, through the consumption of ammonium as nitrogen source. During the time

when the growth of *E. coli* was oxygen-limited, acetate was produced, thus, reduced the pH-level down to 6.5. Once the acetate was consumed, the pH increased again to a value of 6.8.

In the cultivation presented in Fig. 4-3b, 0.1 M MOPS buffer was used to demonstrate the growth of *E coli* BL21 pRSET eYFP-IL6 with a buffer concentration that was 50% less than that in the previous experiment (Fig. 4-3a). The OTR indicated an exponential growth phase until ca. 4 h with an OTR maximum of 0.066 mol/L/h. Then a short plateau was apparent, which indicates oxygen-limited growth. Thereafter, the OTR decreased slowly, until the microorganisms stop to grow after 6 h. This slow decrease of the OTR curve indicated a pH-range suboptimal for growth (Anderlei and Büchs 2001). The pH dropped very fast during the cultivation until it reached its final value of ca. 4.66 after 8 h, when the pH was too low for microbial growth. On the basis of the slow decreasing OTR after 5h, the pH-curve and the reduced increase in the OD-values, it could be concluded that the metabolism of *E. coli* pRSET eYFP-IL6, with the applied conditions, is reduced at pH-values below 5.8. Here, the pH-value dropped below 5.8 at 5 h, while the OTR decreased and the growth was reduced and finally stopped, although the glucose was not yet depleted. During the oxygen limitation, the microorganisms started to produce acetate up to a concentration of 2.3 g/L. Even though the carbon source glucose was completely exhausted at ca. 7 h, the produced acetate was not consumed and no metabolic activity and no growth could be observed; this means that the very low pH in the medium completely hampered the growth of the microorganisms.

To demonstrate the applicability of the controlled-release of sodium carbonate for controlling the pH, an experiment with buffer in a concentration of 0.1 M MOPS and three controlled-release systems (30% (w/w) sodium carbonate content) was performed (Fig. 4-3c). Here, the OTR curve followed the same shape as in the reference cultivation with 0.2 M MOPS buffer depicted in Fig. 4-3a. In the beginning, the OTR exhibited exponential growth of the microorganisms until ca. 4 h, followed by a period of ca. 1 h of oxygen-limited growth with a maximum OTR value of 0.063 mol/L/h. While the culture was oxygen-limited, 1.79 g/L acetate was produced. The glucose concentration decreased continuously until it was completely consumed after about 5.8 h. Once the glucose was depleted, the OTR first decreased sharply and then peaked again to 0.034 mol/l/h after ca. 7.5 h. In this case, the microorganisms completely consumed the previously produced acetate in the diauxic growth phase. After about 9.5 h, acetate was also exhausted. Due to the continuously released sodium carbonate (see Fig. 4-1) and the low biomass concentration, the pH increased in the beginning

of the cultivation up to a value of 7.66 after ca. 3 h. Thereafter, the pH curve decreased to a value of 6.33 until ca. 6 h cultivation time. This corresponds to the peak of acetate concentration. During this interval, the biomass concentration rose and more protons were produced by the microorganisms than sodium carbonate was released from the controlled-release systems. Once the glucose was depleted and replaced by acetate as carbon source the pH increased. This increase showed a steeper slope than in the cultivation with 0.2 M MOPS buffer, because sodium carbonate is still additionally released continuously from the controlled-release systems and the medium contains less buffer to prevent excessive pH-drifts.

These experiments proved that the pH-control in shake flasks with controlled-release of sodium carbonate works. With this technique it was possible to halve the buffer concentration in the Wilms-MOPS medium with 20 g/L glucose and to establish the same growth behavior such as that with the higher reference buffer concentration (0.2 M MOPS buffer).

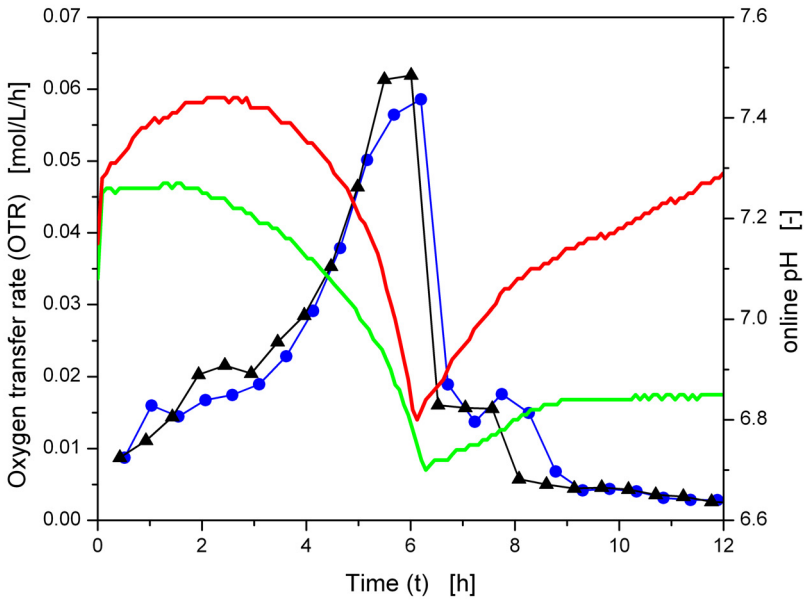

Fig. 4-4: Comparison of oxygen transfer rate (OTR) and online pH of *E. coli* BL21 pRSET eYFP-IL6 during growth in Wilms-MOPS medium with different buffer concentrations and controlled-release systems. With 0.2 M MOPS buffer OTR (●) and pH (—), with 0.1 M MOPS buffer and 3 controlled-releases systems, OTR (▲) and pH (—). Experimental conditions: Wilms-MOPS medium with 20 g/L glucose, 37°C; 10 mL filling volume; shaking diameter (d_0) 50 mm; 350 rpm; $OD_{600,\alpha}$ = 0.5; pH_0 = 7.5; controlled-release systems: D = 15 mm; H = 1.1 mm; 30% (w/w) Na_2CO_3.

For validating this successful application of the controlled-release system and to measure the whole pH-course, *E coli* BL21 pRSET eYFP-IL6 was cultivated in a RAMOS device with integrated online pH measurement (see chapter 3). One fermentation with 0.2 M MOPS buffer

concentration and one with 0.1 M MOPS and three additional controlled-release systems were conducted (Fig. 4-4). The OTR curves of both cultivations followed nearly the same course. Both curves showed the first peak while glucose was being consumed as carbon source. Then, after ca. 6 h both curves plummeted, due to the depletion of glucose, before the microorganisms switched to acetate as carbon source and a second peak was formed. In the beginning of the cultivation with a MOPS buffer concentration of 0.2 M, the pH did not change because of the high buffer capacity of the medium and the relatively low metabolic activity of the microorganisms. During the exponential growth phase of the microorganisms the pH decreased. Subsequently, the pH increased during the time when the acetate was consumed in the diauxic growth phase.

The pH of the culture with 0.1 M MOPS buffer and three controlled-release systems rose in the beginning of the experiment to a maximum value of ca. 7.7 due to the constant release of sodium carbonate (refer to Fig. 4-1). After ca. 2.5 h, the increased microbial activity yielded more protons compared to the amount of sodium carbonate released. Thus, the pH decreased to a minimal value of ca. 6.3 until the glucose was depleted from the medium. In the diauxic growth phase the pH increased rapidly because of the consumption of acetate and the continuing release of sodium carbonate. This sodium carbonate release also resulted in still slightly increasing pH-values during the stationary growth phase of the culture. These results clearly demonstrated that the application of the controlled-release systems was successful for controlling the pH in *E. coli* BL21 pRSET eYFP-IL6 cultures with 20 g/L glucose in Wilms-MOPS medium. Furthermore, the buffer concentrations in the medium could be substantially reduced, thus, improving the comparability of small-scale shake flask cultures with large-scale fermentations.

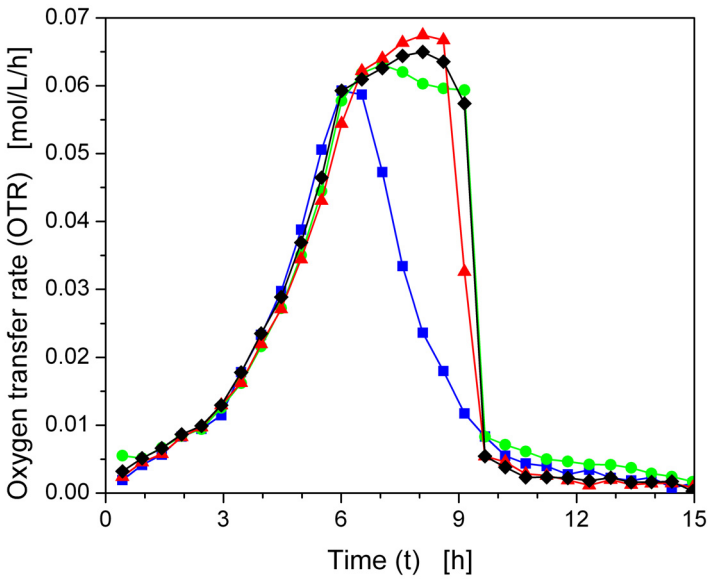

Fig. 4-5: Oxygen transfer rate (OTR) of *E. coli* K12 during growth in Wilms-MOPS medium with different buffer concentrations, initial pH-values and controlled-release systems. With 0.2 M MOPS buffer, initial pH 7 (■), 0.2 M MOPS buffer, initial pH 7.5 (◆), with 0.1 M MOPS buffer, initial pH 7 and 3 controlled-releases systems (●), with 0.1 M MOPS buffer; initial pH 7.5 and 3 controlled-releases systems (▲). Experimental conditions: Wilms-MOPS medium with 20 g/L glycerol, 37°C; 10 mL filling volume; shaking diameter (d_0) 50 mm; 350 rpm; $OD_{600,\alpha}$ = 0.5; controlled-release systems: D = 15 mm; H = 1.1 mm; 30% (w/w) Na_2CO_3, washed for 30 min in H_2O.

To demonstrate the application of the sodium carbonate containing controlled-release system with another strain and carbon source, *Escherichia coli* K12 with Wilms-MOPS medium with 20 g/L glycerol was cultivated in varying buffer concentrations and initial pH values (Fig. 4-5). In this experiment, four cultures were compared – two of which were cultivated at initial pH values of 7, whereby one of these was grown with and the other one without controlled-release systems. The remaining two cultures, in contrast, were cultivated at an initial pH-value of 7.5 and, analogously, one of each grown with and without the controlled-release systems.

Previous experiments with glycerol as carbon source revealed that the growth rate of *E. coli* was lower than with glucose as carbon source. For that reason the sodium carbonate release from untreated controlled-release systems was too fast in the beginning. Consequently, the pH increased rapidly to suboptimal high values (data not shown). Therefore, all experiments with glycerol as carbon source used controlled-release systems, which were washed in water for 30 min. During these 30 min, the very fast release of the Na_2CO_3 in the beginning of the release kinetics, as described in Fig. 4-1, can be intercepted.

The culture with an initial pH 7 and in the absence of a controlled-release system (squares) showed the triangular OTR-curve indicating that the pH-value became too acidic for normal metabolic activity (see Fig. 4-2). On the contrary, the culture with the higher initial pH-value of 7.5 (diamonds) reached a higher OTR and entered a plateau after about 6 h. At the end of the plateau, the OTR decreased slowly until ca. 9 h and then dropped sharply. The first slight decrease indicated a suboptimal pH-value, before the glycerol was exhausted. The differences in the two cultures with initial pH-values of 7 and 7.5, respectively is dependent on the buffer capacity of the used MOPS buffer. The pK_a-value of MOPS buffer at 37°C is 6.98 and the pH buffer capacity lies in the range of about +-1 of this pKa-value. Therefore, the experiment with initial pH-value of 7.5 can utilize more of the buffer capacity during the cultivation, than the culture with an initial pH-value of 7.

Using the controlled-release systems, the culture at an initial pH 7 (circles) depicted a much less impaired metabolic activity compared to the respective culture without sodium carbonate release (squares). Only between ca. 7.5 and 9 h a slightly reduced metabolic activity can be seen in the OTR. The usage of an initial pH of 7.5 and addition of controlled-release systems (triangles) resulted in a preferred OTR curve in this experiment. No impact of suboptimal pH was recognizable and only a short oxygen limitation was obvious in the short plateau between 7.5-9 h.

Therefore, an initial pH value of 7.5 is more suitable for *E. coli* K12 under the applied conditions, because the culture can take advantage of the better utilized buffer capacity of the applied medium than the culture with initial pH-value of 7. Furthermore, it is evident that the culture parameters (e.g. the initial pH-value and buffer concentration) have to be chosen very carefully to benefit from the pH-control with the polymer-based controlled-release systems. Without the optimal parameters, for example, the pH could drift very fast into too high or too low values for metabolic activity of the microorganisms.

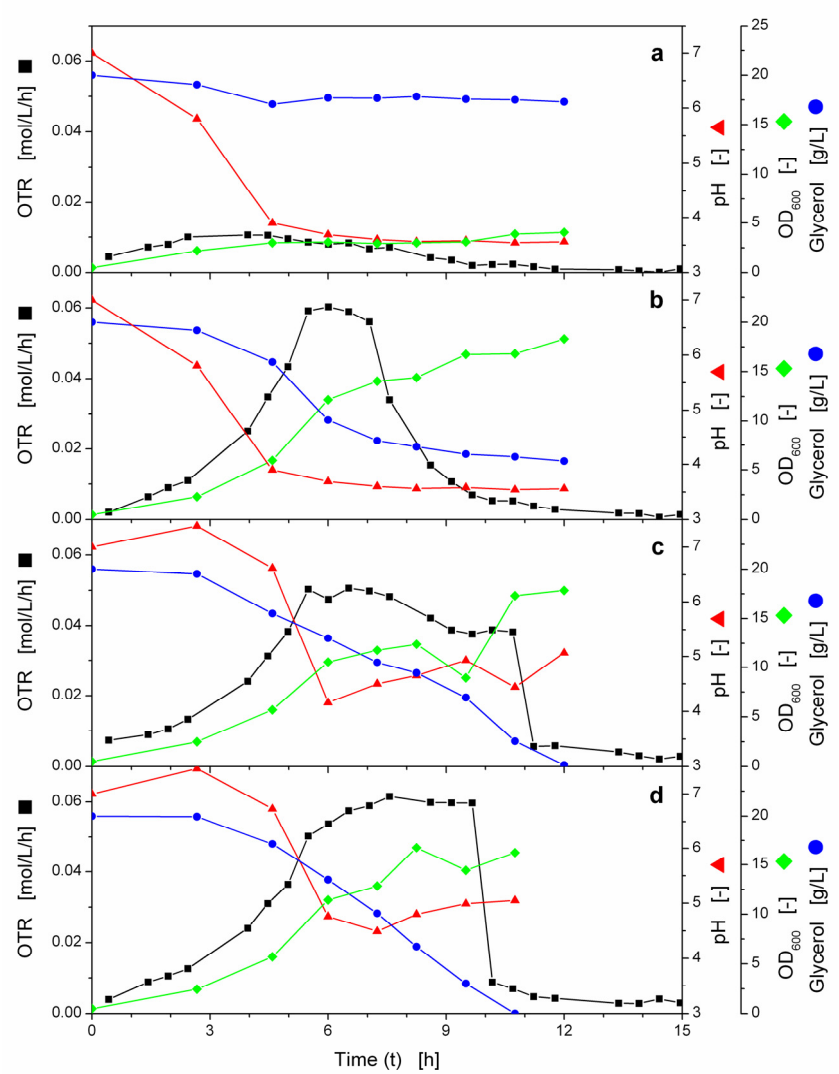

Fig. 4-6: Comparison of the oxygen transfer rate (OTR), pH and glycerol concentration during the cultivation of *E. coli* K12 in buffered or pH-controlled media using controlled-release systems in shaking flasks. a: without buffer, b: with 0.2 M MOPS buffer, c: without buffer and additional 3 controlled release systems, d: without buffer and additional 4 controlled-release systems. OTR (■); pH (▲); glycerol concentration (●); OD_{600} (◆); Experimental conditions: Wilms-MOPS medium with 20 g/L glycerol, 37°C; 10 mL filling volume; shaking diameter (d_0) 50 mm; 350 rpm; $OD_{600,\alpha}$ = 0.5; pH_0 = 7; controlled-release systems: D = 15 mm; H = 1.1 mm; 30% (w/w) Na_2CO_3, washed for 30 min in H_2O.

To prove the applicability of the controlled-release system without any addition of buffer in the medium, experiments were performed in Wilms-MOPS medium with 20 g/L glycerol with and without MOPS buffer and controlled-release systems, respectively. A suboptimal initial pH-value of 7 (see Fig. 4-5) was chosen for these cultivations to demonstrate the functionality of the pH-control using controlled-release systems even under suboptimal conditions. One cultivation was performed without MOPS buffer (Fig. 4-6a). In this experiment the pH decreased rapidly. The pH-value of the medium below 4 hampered the growth of the microorganisms completely. In the second experiment the same conditions were chosen, except for an increased MOPS buffer concentration of 0.2 M (Fig. 4-6b). Applying this

standard buffer concentration, the OTR rose to a value of 0.06 mol/L/h in the beginning, followed by a short plateau indicating oxygen limitation and demonstrating a maximum oxygen transfer capacity for this cultivation conditions. Than the OTR decreased in a triangular shape, indicating that the metabolism of the microorganisms is impaired by too low pH (Anderlei and Büchs 2001). The pH curve decreased during the exponential growth phase and reached a suboptimal value of ca. 3.5 after 8 hours. At the end of the cultivation the carbon source glycerol was not depleted which indicates that the metabolism of *E. coli* K12 was indeed hindered by too acidic conditions.

To investigate the influence of the new controlled-release system, in the third experiment no buffer was used, but three controlled-release systems (Fig. 4-6c). The OTR in this experiment displayed a normal growth of the microorganisms until a maximum was reached at 5 h. Thereafter, the OTR decreased slowly until 11 h when the glycerol was depleted. During this time the culture was not oxygen-limited, because the maximum oxygen transfer capacity of 0.06 mol/l/h, as described in Fig. 4-6b, was not reached in this experiment. This gradual decrease in the OTR curve is attributed to the pH-values between 4.2 and 5 which were partially, however, not sufficiently counterbalanced by the Na_2CO_3 release from the controlled release systems.

In the fourth experiment no buffer and 4 controlled-release systems were investigated (Fig. 4-6d). In this final experiment, an even better metabolic activity could be observed than with three controlled-release systems (Fig. 4-6c). The OTR peaked at a value around 0.06 mol/L/h and formed a plateau for 3 h. This plateau indicates oxygen-limited growth of *E. coli* and no negative influence of too low pH-values could be observed in the growth and the metabolic activity. The complete carbon source glycerol was consumed by the microorganisms. Although the pH-value reached a very low value at 4.5, the metabolic activity is sustained, due to the continuous release of the Na_2CO_3. The pH-curve depicts the most suited pH range for growth of the microorganisms of these four experiments in a range of ca. 4.5 and 7.4. Therefore, the controlled-release systems enable the user to control the pH without any additional buffer. Furthermore, the osmolarity of the medium was dramatically reduced with the abdication of the buffer.

These experiments showed that the controlled-release systems could indeed control the pH-values of the medium, thereby ensuring good microbial growth.

4.4 Conclusion and Outlook

Conclusion

The presented polymer-based controlled-release system embedding sodium carbonate crystals for controlling the pH in shake flasks enabled the successful cultivation of *E. coli* K12 and *E. coli* BL21 pRSET eYFP-IL6 in mineral media with glycerol and glucose as carbon sources, respectively. With the controlled-release system it is possible to substantially reduce the buffer concentrations in media for shake flasks cultures, while the pH-values remain in the physiological range for sustained microbial activity during the whole cultivation. This reduction in buffer concentration leads to reduced osmolarities in the medium. These reduced osmolarities may significantly enhance the growth rates of *E. coli*, as demonstrated in Fig. 4-2 and described by Record et al. (Record et al. 1998).

Large-scale fermentation processes are applied with an active pH-control. In these processes no buffers are used. The here presented polymer-based controlled-release systems enable comparable cultivation parameters in shake flasks such as in large-scale, while the buffer concentrations are dramatically reduced and the pH is controlled in a narrow range. These advantages are very suitable for high-throughput screening experiments, where the reduced osmolarities in the medium enhance the optimal strain selection.

The polymer-based controlled-release system for fed-batch cultivation in shaken bioreactors developed by Jeude et al. (Jeude et al. 2006) is a self-regulating system. During the lag phase of the microorganisms the released glucose accumulates in the medium (see chapter 6). When the biomass concentration increases during the cultivation the accumulated glucose is consumed until the substrate-limited fed-batch phase. In the fed-batch phase, the microorganisms directly consume the released glucose. When different cultivation parameters such as various starting biomass concentrations or lag phases occur in parallel cultures (e.g. precultures for screening experiments), then all cultures enter the fed-batch phase at different points in time. When all cultures are in the fed-batch mode, they have consumed the same amount of glucose and, therefore, their biomass concentrations and their growth rates are equalized dependent on the glucose release kinetics (see chapter 6). In contrast, the in this chapter presented Na_2CO_3 containing polymer-based controlled-release system is not self-regulating. Here, the Na_2CO_3 salt is released and influences the pH-value of the medium, no matter if the microorganisms are growing or not. Additionally, the sodium carbonate is then

not consumed by the microorganisms such as the glucose in the fed-batch system. Therefore, it is very important to ensure optimal growth parameters such as initial pH-values and biomass concentrations while using the newly developed controlled-release system with pre-defined release kinetics. Especially, the lag phase of a culture has a tremendous effect on the performance of the system. For example, when the lag phase is too long, the pH increases very fast to suboptimal high values due to the high initial release of the Na_2CO_3.

Outlook

In our Laboratories controlled-release systems which enhance the release of alkaline compounds, such as Na_2CO_3, in response to reducing pH-values are currently under development. This will lead to complete pH-regulation in small-scale cultures in the future. With such a pH-sensitive controlled release system, it will be possible to cultivate microorganisms in small-scale shaken bioreactors without any additional buffer, thereby ensuring culture conditions in the optimal pH-range.

5. Influence of initial pH-values on the lag phase of *E. coli* batch cultures

5.1 Introduction

The duration of the lag phase of microorganisms is an interesting topic to the food industry; since a longer lag phase of microorganisms in food implies that the shelf-life of food products increases, thereby bolstering profits. Thus, food microbiology, in particular the area of predictive microbiology, is engaged in modelling microbial growth. Several models for the growth of the pathogenic *Escherichia coli* O157:H7 (Buchanan et al. 1993; Li et al. 2006), *Salmonella typhimurium* (Dufrenne et al. 1997) or *Listeria monocytogenes* (Hudson 1994) have been developed. Moreover, the lag phase is also relevant for multiple applications in the field of biotechnology. The factors which influence the lag phase are important for screening processes to ensure a parallel and equal initiation of growth. A shorter lag phase and a faster growth may reduce the duration of cultivation, thus saving time and money. The control of the lag phase is also important to obtain absolutely reproducible and robust production processes in a GMP (Good Manufacturing Practice) environment.

One important factor influencing the growth of *E. coli* is the pH-value of the culture medium; an excessively acidic or alkaline medium inhibits or even terminates growth. The best pH-value for optimal growth of *E. coli* lies in the range of 6.5-7.5. It increases with increasing temperature (Davey 1994; Munro 1970). Presser et al. (1997) stated that the fastest growth rate of *E. coli* M23 ranges from pH 6 to 7.5, whereas later studies indicate a minimum pH-value for uninhibited growth of 5.8 (see chapter 4). *E. coli* itself may affect the pH of the medium, for example, by producing acids and consuming ammonium as a nitrogen source (Christensen and Eriksen 2002). Minimal media, which are typically used in screening experiments because of their defined composition, generally contain ammonium as nitrogen source. Therefore, upon consumption of this nitrogen source, the pH-value inevitably drops during cultivation.

E. coli can tolerate low pH-values for some time. As Benjamin and Datta (1995) showed, the pH-value of the growth medium influences the ability of *E. coli* to survive subsequently in acidic medium. In Benjamin and Datta´s study, *E. coli* was first grown in cultures at pH-values of 5-8. Thereafter, upon being transferred to acidic medium, the *E. coli* from the originally acidic cultures tolerated the new acidic medium better. This acid tolerance of the *E. coli* varied depending on the strain investigated. This effect, however, was observed in all investigated *E. coli* strains; i.e., those grown in acidic medium always tolerated a new acidic medium. Different mechanisms cause this acid tolerance. For example, Brown et al. (1997) reported that the acid tolerance of *E. coli* appears to be correlated to the cyclopropane fatty acid content of the cellular membrane. Acid adapted *E. coli* had cell membranes that differed from those of non-adapted *E. coli.* Furthermore, Jordan et al. (1999) assumed that the acid tolerance of *E. coli* is correlated to the proton-permeability of the bacterial cellular membrane. When the cellular membrane is less permeable to protons, the cytoplasm obviously becomes less acidic. This reduced permeability appeared to be related to a change in protein composition of the membrane. Another mechanism to prevent the acidification of the cytoplasm has been suggested by Hersh et al. (1996). They state that *E. coli* alkalizes the cytoplasm itself by a glutamate decarboxylase alkalinisation cycle. Thus, several different mechanisms allow *E. coli* to grow or survive even in media having suboptimal pH-values and recover later in media with a more suitable pH-value.

In small scale cultivations, which are typically used for screening purposes, buffers are used to maintain an optimal pH-range. There are many different buffers, which can be used to minimize a shift of the pH-value during bacterial cultivation. A buffer can only tolerate a given quantity of acid or base, before its capacity is exhausted, causing the pH-value of the medium to rapidly sink or rise. The higher the buffer concentration, the more acid can be neutralized. However, at higher buffer concentrations, the osmolarity of the medium rises. Record et al. (1998) reported that an osmolarity of more or less than 0.3 Osmol/L reduces the growth rate of *E. coli* in minimal medium (see chapter 1.2 and 4). Consequently, both excessive and insufficient concentrations of buffer can adversely affect *E. coli* growth. Besides affecting the growth rate of *E. coli*, the pH-value may also influence the actual lag phase before growth starts. Consequently, the aim of this chapter is to determine the influence of the initial pH-value on the lag phases of four different *E. coli* strains.

5.2 Material and Methods

Microorganisms

For this study, the strains *E. coli* K12 (ATCC 23716, DSMZ, Braunschweig, Germany), *E. coli* BL21 pRSET eYFP-IL6 (referred to in this chapter as *E. coli* eYFP) (Samorski et al. 2005), *E. coli* SCS1 pQE-30 pSE111 (referred to in this work as *E. coli* PR02) (Büssow et al. 1998) and *E. coli* BL21(DE3) pRhotHi-2-EcFbFP (referred to in this chapter as *E. coli* EcFbFP) were used. The clone PR02 was kindly provided by Protagen AG, Dortmund, Germany. Moreover, the clone pRhotHI-2-EcFbFP was kindly provided by T. Drepper, Institute of Molecular Enzyme Technology, Heinrich-Heine-University Düsseldorf, Germany. Stock cultures were prepared in a Wilms-MOPS medium (Wilms et al. 2001b) with additional 150 g/L glycerol and were stored in 1 mL aliquots at -80°C.

Media

A modified Wilms and Reuss medium (henceforth referred to as Wilms-MOPS medium) was used for the various cultivations (Jeude 2007; Wilms et al. 2001). It consists of 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 3.0 g/L K_2HPO_4, 2 g/L Na_2SO_4, 0.5 g/L $MgSO_4*7H_2O$, 0.01 g/L Thiamine hydrochloride, 20.9 g/L 3-(N-morpholino)propanesulfonic acid (MOPS) (0.1 M), 10 g/L glucose and 1 mL/L trace element solution. This trace element solution consists of 1.98 g/L $CaCl_2*2H_2O$, 0.54 g/L $CoCl_2*6H_2O$, 0.48 g/L $CuSO_4*5H_2O$, 41.76 g/L $FeCl_3*6H_2O$, 0.3 g/L $MnSO_4*H_2O$, 33.39 g/L Na_2EDTA (Titriplex III), 0.54 g/L $ZnSO_4*7H_2O$. The pH was adjusted with NaOH (5M) to 6.5, 6.75, 7, 7.25 and 7.5, respectively. Furthermore, the osmotic pressure of the main culture medium was balanced. NaCl was added to equalize the osmolarity of the media with its different pH-values. The osmolarity was measured with an osmometer (Gonotec, Berlin, Germany). A concentration of 0.1 g/L ampicillin was added for the cultivation of *E. coli* BL21 eYFP and *E. coli* PR02. All reagents were purchased from Fluka Chemie GmbH (Buchs, Switzerland), Merck (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany) or Sigma Aldrich Chemie GmbH (Crailsheim, Germany).

BioLector experiments

The BioLector system, which detects the biomass concentration of a given solution online and quasi-continuously through measurement of scattered light intensity in shaken microtiter plates, was used (Samorski et al. 2005) (see chapter 1.3). A 620 nm filter was used to measure the scattered light, and black 96-well microtiter plates with a clear bottom (Greiner Bio-one, Frickenhausen, Germany) were applied for the cultivations. For precultivation, 250 mL Erlenmeyer flasks containing 10 mL Wilms-MOPS medium were inoculated with stock cultures. The precultures were cultivated overnight at 37 °C, a shaking frequency of 350 rpm and a shaking diameter of 3 mm.

To determine the influence of the initial pH-value on the lag phase of *E. coli*, parallel experiments with varying pH-values in microtiter plates were carried out. In a first experiment, *E. coli* eYFP was cultivated in Wilms-MOPS medium with five different initial pH-values (6.5, 6.75, 7, 7.25 and 7.5). Main cultures were inoculated with overnight Wilms-MOPS precultures and three different initial OD (0.05, 0.1 and 0.15). They were cultivated at 37 °C, a shaking frequency of 950 rpm, a shaking diameter of 3 mm and a filling volume of 200 µL per well. Their growth was observed with the BioLector system. For every different initial pH-value, every different initial OD was applied in six parallel wells. For another experiment, *E. coli* eYFP, *E. coli* K12 and *E. coli* PR02 were cultivated under the same conditions, but the main cultures were inoculated with an initial OD of 0.1 and every different initial pH-value was applied in five parallel wells.

RAMOS experiments

As described by Anderlei et al. (2004), a self-made RAMOS device was used to perform online measurements of the Oxygen Transfer Rate (OTR) in shake flasks. A commercial version can be purchased from Kühner AG, Birsfelden, Switzerland and HiTec Zang GmbH, Herzogenrath, Germany. The RAMOS cultivations were performed in modified 250 mL Erlenmeyer flasks as described by Anderlei and Büchs (2001). A Wilms-MOPS preculture was inoculated with a stock culture of *E. coli* K12. It was grown for 7.5 h in a modified 250 mL Erlenmeyer flask at 37 °C, a shaking frequency of 350 rpm, a shaking diameter of 50 mm and a filling volume of 10 mL. Wilms-MOPS main cultures were inoculated with this preculture, resulting in an initial OD of 0.1 of the main cultures. They were then grown at 37 °C, a shaking frequency of 350 rpm, a shaking diameter of 50 mm and a filling volume of 10 mL per flask.

Na_2CO_3 controlled release system

To keep the pH-value of a cultivation in a narrow range without using high amounts of buffers, a controlled release system, consisting of polymer-based discs (Jeude et al. 2006) with integrated Na_2CO_3, was applied (see chapter 4). To manufacture the release system, solvent-free two components (called component A and B by the manufacturer) silicone Sylgard™184 as well as the catalyst Syl-off™ 4000 (Dow Corning, Wiesbaden, Germany) in a concentration of 0.1 % (w/w) were used. The ratio between the two components A and B was 10:1 as recommended by the manufacturer. Na_2CO_3 with the highest degree of purity was supplied by Sigma Aldrich (Crailsheim, Germany). The Na_2CO_3 was milled with a vibration micromill (Spartan™, Fritsch, Idar-Oberstein, Germany) in a high-grade steel mortar and then sieved through test sieves (Fritsch, Idar-Oberstein, Germany). The fraction with particle sizes ranging from 20 to 50 µm was used. First, a mixture consisting of component A of Sylgard™184, Na_2CO_3 (30 % (w/w)) and catalyst was weighed and degassed in a desiccator in a 30 mbar vacuum for 0.5 h. Then, component B was added. The finished mixture was casted on a glass plate with a casting knife (gap 1.1 mm) and then cross-linked at 50 °C in a convection oven for 3 h. The applied discs had a diameter of 15 mm (Further details will be published elsewhere). The discs released Na_2CO_3 at a certain rate, so that the medium was alkalized during cultivation, thus counteracting any biological acidification effects. The controlled-release systems were provided by the ITMC of the RWTH Aachen University and the release kinetics are described in chapter 4.

The release system was used for one set of precultures. Three different precultures of *E. coli* eYFP in Wilms-MOPS medium with 0.2 M MOPS buffer were made. One batch was cultivated with one Na_2CO_3 disc, one with two Na_2CO_3 discs and another one without a Na_2CO_3 disc. These overnight precultures were cultivated in 250 mL Erlenmeyer shake flasks at 37 °C, a shaking frequency of 350 rpm, a shaking diameter of 50 mm and a filling volume of 10 mL per flask. Main cultures were inoculated with an initial OD of 0.2 and their growth was observed with the BioLector system. For every different final pH-value of the preculture, every different initial pH-value was applied in five parallel wells.

Determination of the lag phase

The lag phase was estimated as described by Zwietering et al. (1990). Hereby, the logarithm of the biomass divided by the initial biomass was plotted against time. To obtain the lag phase, the intersection point between the x-axis and the partial regression line of the linear span of the growth curve was determined.

Calculation of titration curves

The pH-value of a buffer depends on its pK_A and its proportion of acid and conjugate base. Addition of acid or base to the buffer changes the proportion and thus lowers or increases its pH-value. Whereas small amounts of added acid or base result in a small change in pH, the buffer capacity can be exhausted by adding a larger amount of acid or base. In that case, the pH-value declines or increases rapidly. For a given buffer at a known concentration, its pH-value - dependent on the amount of added acid or base - can be calculated by the Henderson-Hasselbalch equation.

$$pH = pK_A - \log_{10}\left(\frac{c_{HA}}{c_{A^-}}\right) \tag{5-1}$$

Calculation of produced acid

The course of pH during cultivation can be calculated by Equation 5-2 (Stöckmann et al. 2003b).

$$pH = pK_A - \log_{10}\frac{c_{HA,0} + c_{H^+}}{c_{A^-,0} - c_{H^+}} \tag{5-2}$$

With known total buffer concentration c_{HA+A^-}, the initial acid and base concentration are

$$c_{HA,0} = \frac{10^{pK_A - pH_0}}{10^{pK_A - pH_0} + 1} * c_{HA+A^-} \tag{5-3}$$

and

$$c_{A^-,0} = \frac{1}{10^{pK_A - pH_0} + 1} * c_{HA+A^-} \tag{5-4}$$

respectively. Insertion of Equation 5-3 and 5-4 into Equation 5-2 yields Equation 5-5.

$$pH = pK_A - \log_{10} \frac{\frac{10^{pK_A - pH_0}}{10^{pK_A - pH_0} + 1} * c_{HA+A^-} + c_{H^+}}{\frac{1}{10^{pK_A - pH_0} + 1} * c_{HA+A^-} - c_{H^+}} \quad (5\text{-}5)$$

Equation 5-5 was applied, to calculate the amount of acid produced during cultivation. Solving Equation 5-5 for c_{H^+} results in a rough estimate of the amount of acid generated at a given pH-value.

5.3 Results and Discussion

Influence of the initial pH-value on the lag phase of *E. coli* BL21 eYFP

Figures 5-1 A and 5-1 B show the scattered light intensity measured for the wells inoculated with an initial OD of 0.05 and 0.15, respectively. Due to a higher initial OD, the curves of Figure 5-1 B peak earlier, than those in Figure 5-1 A. In Figure 5-1 A and 5-1 B, the respective plateaus were reached sooner at an initial pH of 6.5 than at an initial pH of 7.5. The curves of the cultures with an initial pH-value of 7 are located between the curves of pH 6.5 and 7.5 at an initial OD of 0.05 as well as 0.15.

Based on the scattered light intensity curves, the lag phases of the different cultures were determined. The shortest lag phase for each OD appeared at an initial pH-value of 6.5, the longest lag phase at pH 7.5 (Fig. 5-1 C). For cultures with an initial OD of 0.05, the lag phase of cultures grown at an initial pH-value of 6.5 was 1.9 h shorter than that of cultures grown at an initial pH-value of 7.5. These lag phases were 1.7 h and 1.6 h shorter for cultures with an initial OD of 0.1 and 0.15, respectively. To summarize: a higher initial pH-value resulted in a longer lag phase; a lower initial pH-value resulted in a shorter lag phase.

The final pH-value is also depicted in Figure 5-1 C. During cultivation, the media acidified because of ammonium consumption and production of acid. Whereas the final pH-values of the cultures with an initial pH-value of 6.75 are about 5.8, the cultures with higher initial pH-values have final pH-values in a range of 6.2-6.8. The lowest final pH-values appear at the cultures with an initial pH-value of 6.5: They are 3.6, 4.5 and 4.8 for the cultures with an

initial OD of 0.05, 0.1 and 0.15, respectively. An initial pH-value of 6.5 leads, therefore, not only to a shorter lag phase, but also to pH-values below the pH-limit for uninhibited growth during cultivation (pH 5.8).

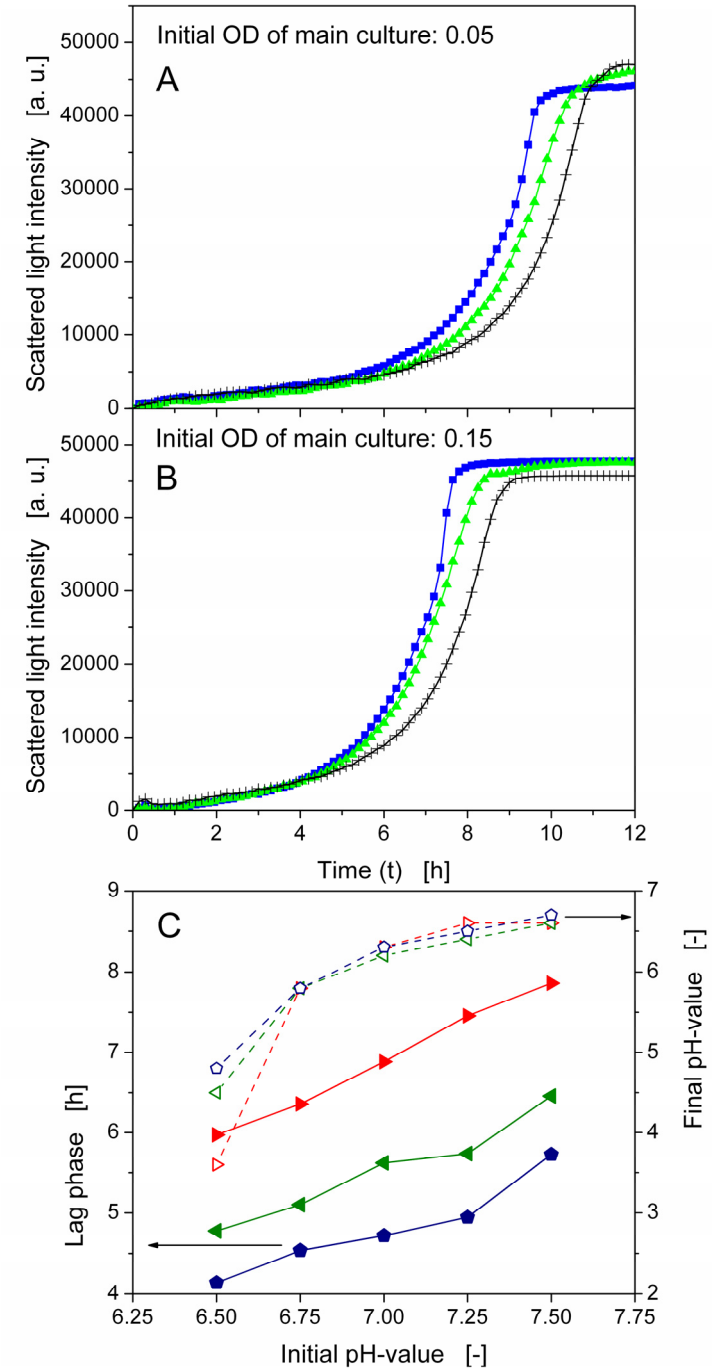

Fig. 5-1: Scattered light intensity [a. u.] (mean of six parallel wells each) of *E. coli* eYFP in microtiter plate with different initial OD and pH-values. A) Initial OD 0.05, B) Initial OD 0.15. Initial pH: (■) 6.5; (▲) 7; (+) 7.5. C) Lag phase (filled symbols; mean of six parallel wells each) and final pH-values (open symbols; mean of six parallel wells each) of *E. coli* eYFP in microtiter plate with different initial pH-values and different initial OD of the main cultures of: (▷,▶) 0.05; (◁,◀) 0.1; (⬠,⬟) 0.15. Experimental conditions: Wilms-MOPS medium with 0.1 M MOPS-buffer and 10 g/L glucose, filling volume per well: 200 µL, shaking frequency (n): 950 rpm, shaking diameter (d_0): 3 mm, temperature: 37 °C, 96-well microtiter plate with gas-permeable seal.

Influence of the final pH-value of the preculture

The aforementioned shorter lag phases at lower initial pH-values might have been caused by *E. coli* adapting to mildly acidic pH-values (Benjamin and Datta 1995). This adaptation to lower pH-values could have occurred during precultivation, which was also carried out in Wilms-MOPS medium with mildly acidic final pH-values. The *E. coli* in the main culture could have had a shorter lag phase, if the initial pH-value of the main culture was in the same pH-range as the final pH-value range of the precultures. To test the influence of the final pH-value of the preculture, three different precultures with differing final pH-values were applied. The pH-values of two special precultures were affected by using a Na_2CO_3 release system, whereas the pH of another standard preculture was not affected. The final pH-values of the two affected cultures were 7.89 (two Na_2CO_3 discs) and 7.55 (one Na_2CO_3 disc), the final pH-value of the non-affected culture was 7.01.

Figure 5-2 A depicts the growth of the *E. coli* cultures inoculated with the preculture having a final pH-value of 7.55. Although the preculture had a mildly alkaline final pH-value, the main culture, with an equally alkaline initial pH-value of 7.5, showed its maximum scattered light intensity at ca. 9 h, whereas the culture having an initial pH-value of 6.5 showed its maximum the earliest, i.e. at ca. 7.5 h. Regardless of the final pH-value of the preculture, the shortest lag phase can be observed at an initial pH-value of 6.5 and the longest at an initial pH-value of 7.5 (Fig. 5-2 B). Even though the precultures with Na_2CO_3 release systems had final pH-values in the mildly alkaline range, the main cultures with mildly acidic initial pH-values (6.5 and 6.75) had the shortest lag phases. Therefore, it is unlikely that an adaptation of *E. coli* to the pH-value during precultivation would be the reason for the shorter lag phase at lower pH-values.

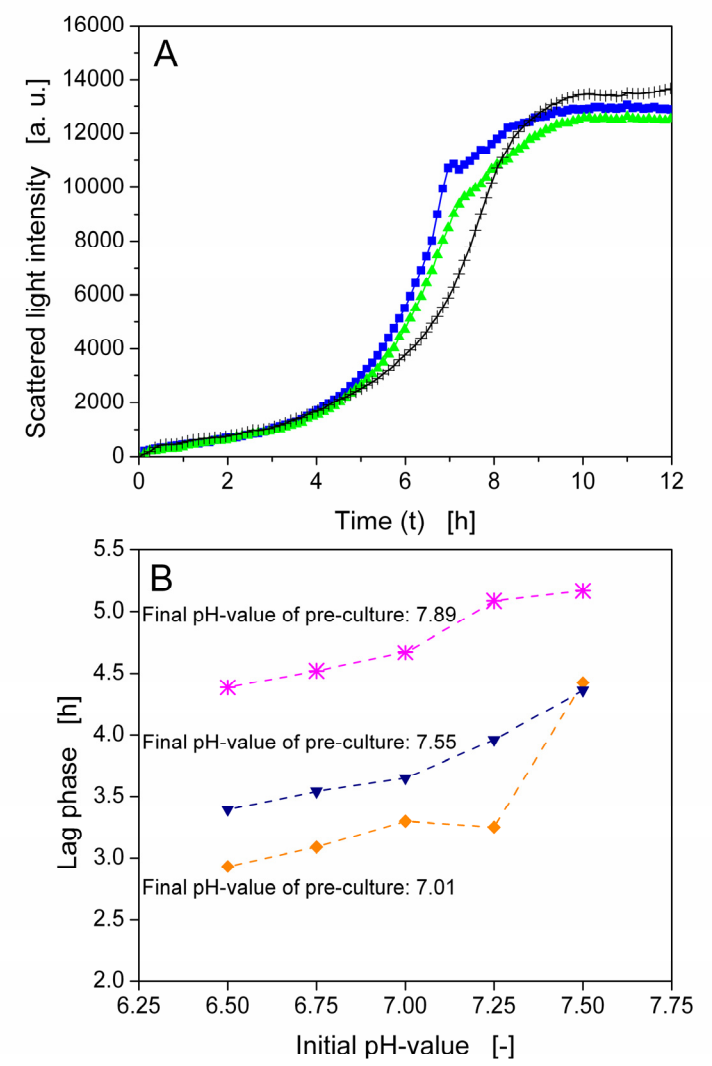

Fig. 5-2: Influence of different initial pH-values on growth and lag phase of *E. coli* eYFP. A) Scattered light intensity [a. u.] (mean of five parallel wells each) of *E. coli* eYFP in microtiter plate with different initial pH-values: (■) 6.5; (▲) 7; (+) 7.5. Final pH-value of the preculture applying controlled release systems with Na_2CO_3 was 7.55. OD_{t0}: 0.2. B) Lag phase [h] of *E. coli* eYFP in microtiter plate with different initial pH-values of the main culture and different final pH-values of the precultures of: (✳) 7.89, (▼) 7.55, (◆) 7.01. Experimental conditions: Wilms-MOPS medium with 0.1 M MOPS-buffer and 10 g/L glucose, filling volume per well: 200 µL, shaking frequency (n): 950 rpm, shaking diameter (d_0): 3 mm, temperature: 37 °C, 96-well microtiter plate with gas-permeable seal.

As stated by Slonczewski et al. (1981), the intracellular pH-value of *E. coli* is 7.6 +/- 0.2 on a range of external pH-values of about 5.5 to 9. A lower external pH-value leads to a higher ΔpH, which they define as the difference between the external pH-value of the medium and the internal pH-value of the cytoplasm. As this difference in pH is involved in the formation of the proton electrochemical gradient (Zilberstein et al. 1984), a lower external pH-value and, hence, a higher ΔpH might positively affect the growth of *E. coli* (Stancik et al. 2002). Calik et al. (2006) also assumed that the *E. coli* growth is inhibited by an extracellular pH greater than or equal to the internal pH-value. Thus, the higher ΔpH might influence the

energy generation, the initiation of metabolic activity, and, therefore, the duration of the lag phase.

Influence of pH on lag phases of different *E. coli* strains

Besides *E. coli* eYFP, two other *E. coli* strains were cultivated to study the influence of the initial pH on the lag phase: i.e. *E. coli* K12 as a laboratory strain and *E. coli* PR02 as another production strain. Whereas *E. coli* K12 and *E. coli* eYFP showed similar behavior, *E. coli* PR02 varied in its response to initial pH-value.

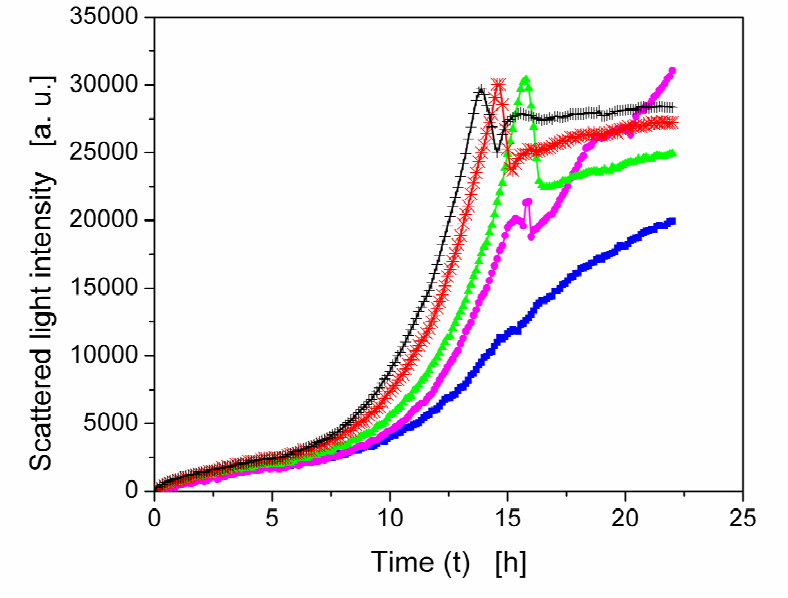

Fig. 5-3: Scattered light intensity [a. u.] (mean of five parallel wells each) of *E. coli* PR02 in microtiter plate with different initial pH-values. (■) 6.5; (●) 6.75; (▲) 7; (✳) 7.25; (+) 7.5.OD_{t0}: 0.1. Experimental conditions: Wilms-MOPS medium with 0.1 M MOPS-buffer and 10 g/L glucose, filling volume per well: 200 µL, shaking frequency (n): 950 rpm, shaking diameter (d_0): 3 mm, temperature: 37 °C, 96-well microtiter plate with gas-permeable seal.

As shown in Figure 5-3, at an initial pH-value of 6.5, the *E. coli* PR02 cultures exhibited restricted growth whereas those with an initial pH-value of 7.5 reached its maximum scattered light intensity the earliest. At an initial pH-value of 7, 7.25 and 7.5 the curves of the *E. coli* PR02 cultures decrease sharply after reaching the maximum scattered light intensity. This decline may be caused by morphological changes of the bacteria. Whereas the maximum scattered light intensity is almost the same for the cultures at an initial pH-value of 7-7.5, the subsequent decline of the various curves differ in magnitude. The lowest decrease in scattered light intensity can be observed at pH 7.5 and the highest decrease at pH 7. Up to about 15 h, the curve of the cultures having an initial pH-value of 6.75 shows a smaller decrease in

scattered light intensity after reaching a first maximum than the aforementioned three cultures. This decrease is followed by a further increase in scattered light intensity. A disadvantageous pH-value accompanied by morphological changes of *E. coli* might explain the curve progression of the cultures with an initial pH-value of 6.75. For the cultures having an initial pH-value of 6.5, the curve progression differs from those of the other cultures: generally, the growth is slower and an exponential phase is hardly detectable. Consequently, the low pH-value adversely affected the growth of this culture initially grown at pH 6.5.

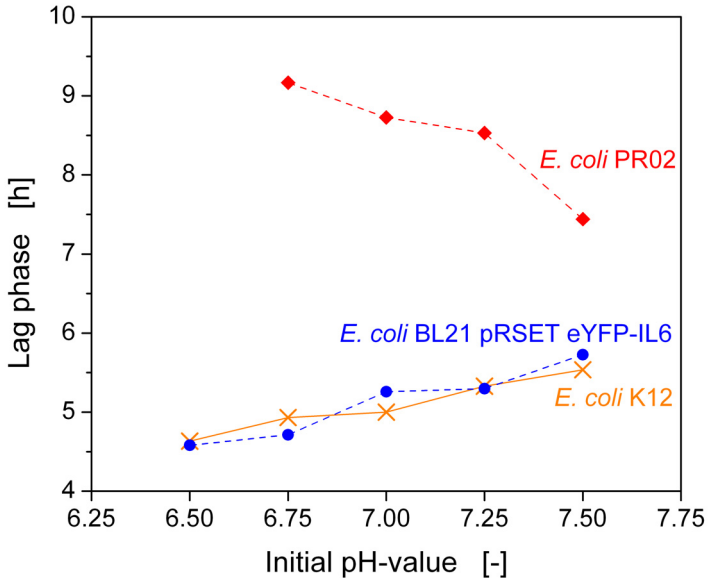

Fig. 5-4: Lag phase [h] (mean of five parallel wells each) of different *E. coli* strains in microtiter plate with different initial pH-values. OD_{t0}: 0.1; (◆) *E. coli* PR02; (●) *E. coli* eYFP; (×) *E. coli* K12. Experimental conditions: Wilms-MOPS medium with 0.1 M MOPS-buffer and 10 g/L glucose, filling volume per well: 200 µL, shaking frequency (n): 950 rpm, shaking diameter (d_0): 3 mm, temperature: 37 °C, 96-well microtiter plate with gas-permeable seal.

The lag phases of *E. coli* K12, *E. coli* eYFP and *E. coli* PR02 are depicted in Figure 5-4. Unlike *E. coli* K12 and *E. coli* BL21 eYFP, *E. coli* PR02 - cultivated in Wilms-MOPS medium having an initial pH-value of 7.5 - demonstrates the shortest lag phase, whereas growth of the culture having an initial pH-value of 6.5 is impaired. Since the exponential phase of the latter is hard to determine, it was impossible to estimate the lag phase of this culture. The lag phase lasts about 9.2 h at an initial pH of 6.75 and ca. 6.4 h at an initial pH of 7.5. The lag phase of *E. coli* PR02 is generally longer than the lag phases of the other strains. On the contrary, for *E. coli* eYFP and *E. coli* K12, the lag phase increases from about 4.6 h to 5.7 h and 5.5 h, respectively. The varying effects of the initial pH-value on the different strains indicate a strain-specific influence of the initial pH-value on the duration of the lag phase. Therefore, in each case the pH-value should be taken into account with regard to the

applied strain. Furthermore, another *E. coli* strain with a different vector, *E. coli* EcFbFP, showed similar results as the strains *E. coli* K12 and *E. coli* eYFP under different cultivation conditions. The lag phase rose from initial pH-values of 7.2-7.8 (data not shown).

RAMOS cultivation of *E. coli* K12

To further study the behavior of *E. coli* in media having different initial pH-values, *E. coli* K12 was cultivated in shake flasks. The oxygen transfer rate (OTR) of *E. coli* K12 was measured using a RAMOS device (Fig. 5-5).

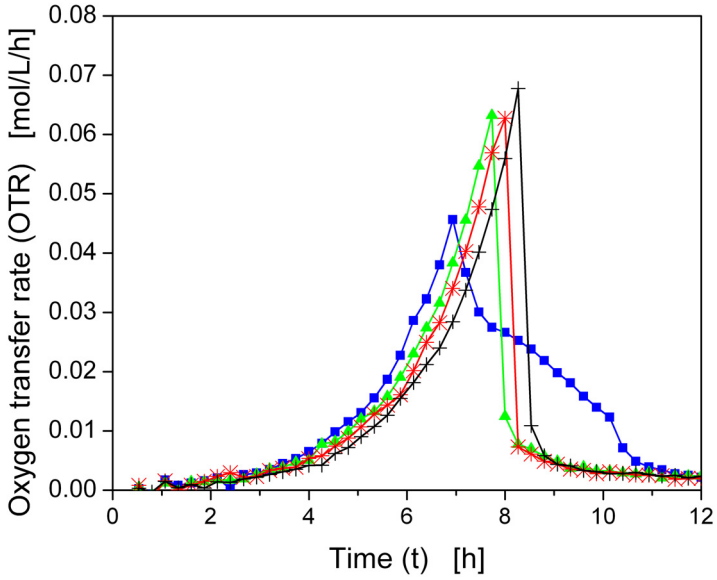

Fig. 5-5: Oxygen transfer rate [mol/L/h] of *E. coli* K12 during growth in 250 mL RAMOS flasks with different initial pH-values. (■) 6.5; (▲) 7; (✳) 7.25; (+) 7.5. OD_{t0}: 0.1. Experimental conditions: Wilms-MOPS medium with 0.1 M MOPS-buffer and 10 g/L glucose, filling volume per flask: 10 mL, shaking frequency (n): 350 rpm, shaking diameter (d_0): 50 mm, temperature: 37 °C.

The cultivations having initial pH-values of 7, 7.25 and 7.5 reached maximum oxygen transfer rates of about 0.07 mol/L/h and declined rapidly in the stationary growth phase. The culture having an initial pH of 6.5 depicts a different behavior, whereby this culture is the first to reach its maximum OTR of approx. 0.045 mol/L/h. Subsequently, this curve declines rapidly to 0.03 mol/L/h and then drops steadily. Unlike the other cultures, this curve progression of the culture at initial pH 6.5 can be explained by the decreasing pH-value of the medium. The medium acidified during cultivation until pH-values that were suboptimal for *E. coli* growth were reached, thus, leading to diminished respiratory activity of *E. coli*. As the medium became more and more acidic, the OTR dropped further until it reached the same level as the other cultures after about 12 h. The final pH-value of the medium was about 3.8.

The other cultures also became increasingly acidic, but to a lesser degree. In these cases, the final pH-values were 6.1, 6.5 and 6.7 for the cultures with an initial pH-value of 7, 7.25 and 7.5, respectively. The OTR curves, displayed in Fig. 5-5 affirm the results of the previous experiments. The higher the initial pH-value is, the later the curves reach their maximum OTR.

Buffer capacity at different initial pH-values

Although the applied medium was buffered with 0.1 M MOPS buffer, the final pH-values of the *E. coli* eYFP cultures having an initial pH-value of 6.5 were about 4 and that for the cultures with an initial pH-value of 7.5 were about 6.7. Ultimately, the production of acetic acid and the consumption of NH_4^+ led to acidification of the medium during the cultivation of *E. coli*.

Figure 5-6 shows the calculated titration curve of the applied 0.1 M MOPS buffer at different initial pH-values. The horizontal dashed line represents the pH-limit (5.8) for uninhibited growth of *E. coli*. At pH-values below 5.8, the growth of *E. coli* is strongly hampered (unpublished data). The vertical dashed line marks the total amount of H^+ produced during cultivation of *E. coli* eYFP in Wilms-MOPS medium with a pH-value of 7.5 under the aforementioned experimental conditions. This amount was calculated with Equation 5-5 and accounted for about 0.043 M H^+. Depending on the initial pH-value of the buffer the theoretically added acid might exceed the buffer capacity, thus leading to pH-values below the limit for uninhibited growth of *E. coli*. This is the case at an initial pH of 6.75 and, in particular, at an initial pH-value of 6.5. Both initial pH-values are below the pK_A= 6.98 of MOPS buffer at 37°C. Thus, at these initial pH-values only a small part of the buffer capacity can be utilized, meaning that a relatively small amount of theoretically added acid exhausts the buffer capacity.

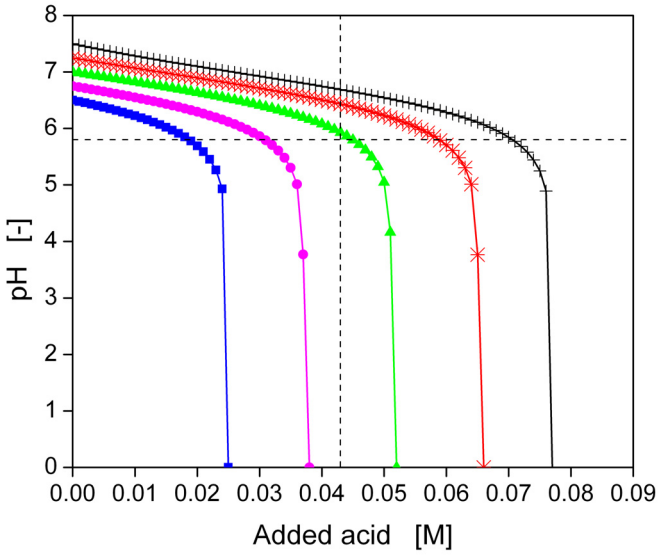

Fig. 5-6: Titration curve of 0.1 M MOPS buffer, calculated with the Henderson-Hasselbalch equation (Eq. 5-1), at different initial pH-values. (■) 6.5; (●) 6.75; (▲) 7; (✳) 7.25; (+) 7.5. Horizontal dashed line: lowest pH-value (5.8) for uninhibited growth of *E. coli* (unpublished data); vertical dashed line: estimated amount of H^+ produced (0.043 M) under the following experimental conditions: Wilms-MOPS medium with 0.1 M MOPS-buffer (pH 7.5) and 10 g/L glucose, filling volume per well: 200 µL, shaking frequency (n): 950 rpm, shaking diameter (d_0): 3 mm, temperature: 37 °C, calculated with Eq. 6-5.

By considering the duration of the lag phase of *E. coli* K12 and *E. coli* eYFP and the titration curve shown in Figure 5-6, two conflicting aims become apparent. The seemingly beneficial effect of a shorter lag phase at lower initial pH-values is offset by minimal pH-values reached during cultivation on the applied media that ultimately hinder the growth of *E. coli*. The amount of acid produced under the applied experimental conditions is high enough to exhaust the capacity of the applied 0.1 M MOPS buffer, resulting in final pH-values of about 4 for *E. coli* eYFP (initial pH-value 6.5).

5.4 Conclusion

In this chapter, the effect of the initial pH-value on the lag phase of four *E. coli* strains in a buffered minimal medium was investigated. For *E. coli* K12 and *E. coli* eYFP as well as *E. coli* EcFbFP, a lower initial pH-value (ranging from 6.5-7.5 for the first two strains and 7.2-7.8 for the third strain) resulted in a shorter lag phase, whereas *E. coli* PR02 showed the opposite behavior. Therefore, the influence of the initial pH-value seems to be strain-dependent. In contrast to the beneficial shorter lag phase of *E. coli* K12, *E. coli* eYFP and *E. coli* EcFbFP, the increasing acidification of the respective media during the cultivation eventually hampered *E. coli* growth. This effect could be avoided by cultivating at a higher

initial pH-value which, in turn, would lengthen the lag phase. Increasing the buffer concentration could be one solution to this dilemma, although a higher buffer concentration would cause a higher osmotic pressure with a potentially adverse impact on the growth of *E. coli*.

Another option would be to use a buffer with a lower pK_A-value (pK_A = 6.25-6.5) than the pK_A-value of MOPS buffer (pK_A = 6.98, 37 °C). As the applied mildly acidic initial pH-values would then lie in the range of the highest buffer capacity, its effectiveness would be increased. Further studies should, therefore, focus on optimizing the applied media and determining the biological causes for the effect of the initial pH-value on the lag phase in various *E. coli* strains.

Furthermore, for process development, the potential benefits from shorter lag phases could be exploited, if the reaction of the applied *E. coli* strain on initial pH-values is taken into account. A future protocol for large-scale cultivation could include, for example, a low initial pH-value and, therefore, a shorter lag phase. At the onset of exponential growth, the pH-value of the medium could be increased by adding alkalizing reagents, thus controlling the pH at optimal values for growth and product formation. The presented controlled-release system (see chapter 4) for controlling the pH could be used for optimizing the initial conditions of small-scale screenings. Here, the initial pH-value of the medium could be set to a low value, resulting in a short lag phase and with the continuous release of the sodium carbonate the pH-value increases during the cultivation to optimal values for growth of the microorganisms.

6. Equalizing growth in high-throughput small-scale cultivations via precultures operated in fed-batch mode

6.1 Introduction

In the post-genomic era, clone libraries are used for many important applications such as screening for drug candidates, efficient new biocatalysts or secondary metabolites as well as media and strain selection (Kumar et al. 2004). The screening of these clone libraries is mainly conducted in shake flasks and microtiter plates in batch mode. In particular, projects in structural genomics, structural proteomics and directed evolution applications are using microtiter plates for high-throughput cultivation of clones for expression studies (Berrow et al. 2006; Graslund et al. 2008; Heddle and Mazaleyrat 2007)).

As described in chapter 1.2 an often underestimated problem when working with different clones in microtiter plates is the non parallel and non equal growth of batch cultures. These growth differences are caused by variances of individual clones regarding, for example, initial biomass concentrations, lag phases or specific growth rates. The non parallel growth in precultures can have a tremendous effect on the performance of bioprocesses. As an example, the process of inducible protein expression will be discussed in more detail with respect to unequal growth kinetics in precultures. In such processes, the addition of an inducer at a predefined time point is the most common way to initiate recombinant protein production. It is well known that inducing at different metabolic states or phases of a culture is a critical factor regarding protein yield (Donovan et al. 1996; Jenzsch et al. 2006). Studier extensively described the problem of simultaneously inducing protein expression of different clones and developed thus an autoinduction medium (Studier 2005). This medium is a highly sophisticated way to cope with the problem of different induction points; however, it cannot be applied to all microorganisms and host/vector combinations. Furthermore, Studier emphasizes that it is very difficult in high-throughput screening to obtain all of the cultures in

a comparable state of growth. One strategy for achieving uniform conditions is to cultivate the organisms until the stationary growth phase (saturation) (Studier 2005). In chapter 1.2 it also was discussed that the growth of precultures to the stationary phase in batch mode may have negative effects on the following main cultivation and therefore, product formation. Additionally, in stationary phase the possible proteolytic degradation of target proteins expressed via IPTG induction or autoinduction may adversely affect product yield (Graslund et al. 2008). It is also known that the inoculum history is very important for the whole main cultivation process regarding reproducibility of growth kinetics (Ferenci 1999; Neves et al. 2001; Webb and Kamat 1993). Therefore the precultivation strategy is very important for a successful screening and it is questionable if the conventional precultivation approaches are the optimal methods for screening for the best producing clones.

Batch cultivations are predominately applied for small-scale cultures because of their easy use, flexibility, low cost and lack of alternative methods. However, fed-batch mode would often be superior for producing biomass and product in main cultures. Furthermore, the fed-batch mode provides more defined physiological conditions and is more often applied in industrial scale than the batch mode. Jenzsch et al. (2006) presented the concept of greatly improving the reproducibility of main cultivations in stirred tank reactors via initiating a fed-batch mode already very early in the fermentation. Reproducibility is of utmost importance especially for good manufacturing practice (GMP), as recommended in the PAT initiative from the FDA (Jenzsch et al. 2006). The authors showed that using fed-batch mode starting in the early biomass formation phase can lead to identical growth profiles of differently inoculated main cultures because of the fixed feed profile (Jenzsch et al. 2007). This concept which Jenzsch et al. proposed for the early phase of a main cultivation served as a basis for equalizing growth in small-scale precultivations with fed-batch mode. The fed-batch mode can be used for different precultures with diverse growth parameters. Therefore, a fed-batch preculture could improve the comparability and the selection of clones in screening applications.

Fed-batch systems on a microliter-scale for screening and bioprocess development are increasingly being developed. These techniques comprise automated stirrer-driven microbioreactors (Puskeiler et al. 2005), microfluidic chips (Leeuwen van 2008) or fed-batch in shake flasks (Ruottinen et al. 2008; Weuster-Botz et al. 2001). As these kinds of devices

require pumps and additional equipment, they are somewhat impractical for fed-batch in real high-throughput.

In this chapter a novel method for equalizing growth kinetics in high-throughput precultures in shake flasks and microtiter plates applying fed-batch mode is presented. For the fed-batch cultivations the polymer-based controlled-release systems for glucose feeding in shake flasks described in chapter 1.4 are used. For fed-batch cultivation in MTPs a newly developed controlled-release system wit hat the bottom of each well immobilized controlled-release system is presented. The focus of this chapter is not the expression of any product directly, but on the growth equalization of precultures. *Escherichia coli* and *Hansenula polymorpha* are used as model organisms.

6.2 Material and Methods

Organisms

E. coli BL21 pRset eYFP-IL6 was maintained in glycerol stocks at -80°C in LB medium with 100 µg/mL ampicillin. This strain was described by Samorski et al. (2005) with an additional plasmid pLysS. *Hansenula polymorpha* RB11 pC10-FMD (P_{FMD}-GFP) (Amuel et al. 2000; Gellissen 2000) was maintained in glycerol stocks at -80°C in YNB medium and was kindly provided by Dr. C. Amuel (Heinrich-Heine University, Department of Microbiology, Düsseldorf, Germany).

Media and Solutions

Modified Wilms & Reuss synthetic medium (henceforth referred to as Wilms-MOPS medium) was used for the *E. coli* cultivations (Jeude 2007; Wilms et al. 2001). The medium consists of 20 g/L glucose; 5 g/L $(NH_4)_2SO_4$; 0.5 g/L NH_4Cl; 3 g/L K_2HPO_4; 2 g/L Na_2SO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 41.85 g/L 3-(N-Morpholino)-propanesulfonic acid (MOPS); 0.1 g/L ampicillin; 0.01 g/L thiamine hydrochloride; 1 mL/L trace element solution [0.54 g/L $ZnSO_4 \cdot 7H_2O$; 0.48 g/L $CuSO_4 \cdot 5H_2O$; 0.3 g/L $MnSO_4 \cdot H_2O$; 0.54 g/L $CoCl_2 \cdot 6H_2O$; 41.76 g/L $FeCl_3 \cdot 6H_2O$; 1.98 g/L $CaCl_2 \cdot 2H_2O$; 33.39 g/L Na_2EDTA (Titriplex III)]. The pH was adjusted to 7.5 with NaOH.

Hansenula polymorpha was cultivated in Syn6-MES medium. The Syn6-MES mineral medium consisted of 1.0 g/L KH_2PO_4, 7.66 g/L $(NH_4)_2SO_4$, 3.3 g/L KCl, 3.0 g/L

$MgSO_4$•$7H_2O$, 0.3 g/L NaCl, 27.3 g/L 2-morpholinoethanesulfonic acid (MES). This aqueous basic solution was adjusted to pH 6.4. Then the following substances were added (per L basic solution): 6.67 mL calcium chloride solution (150 g/L $CaCl_2$•$2H_2O$), 6.67 mL microelement solution (10.0 g/L $(NH_4)_2Fe(SO_4)_2$•$6H_2O$, 0.8 g/L $CuSO_4$•$5H_2O$, 3.0 g/L $ZnSO_4$•$7H_2O$, 4.0 g/L $MnSO_4$•H_2O, 10.0 g/L EDTA (Titriplex III)), 6.67 mL of vitamin solution (0.06 g/L D-biotin, 20.0 g/L thiamine hydrochloride), 3.33 ml of trace element solution (0.2 g/L $NiSO_4$•6 H_2O, 0.2 g/L $CoCl_2$•$6H_2O$, 0.2 g/L boric acid, 0.2 g/L KI and 0.2 g/L Na_2MoO_4•$2H_2O$). The medium resulted in a final volume of 1023.33 mL and no final pH adjustment was necessary (Gellissen 2004; Jeude et al. 2006).

The medium used for the fed-batch precultivations had no initial glucose because the immediate release of glucose from the controlled-release system renders it unnecessary (Jeude et al. 2006). All reagents were of analytical grade and purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

Manufacture of microtiter plates with controlled-release system

To manufacture microtiter plates with the controlled-release system, denominated as "FeedPlates", solvent-free two-component silicone Sylgard™184 was used. The ratio between the two components was 10:1 as recommended by the manufacturer. Anhydrous glucose was supplied by Sigma Aldrich (Crailsheim, Germany) with the highest degree of purity. The glucose was milled with a vibration micromill (Spartan™, Fritsch, Idar-Oberstein, Germany) in a high-grade steel mortar and then sieved with test sieves (Fritsch, Idar-Oberstein, Germany). The fraction with particle sizes ranging from 20 to 50 µm was used. First, the two silicone components of the Sylgard™184 and glucose were mixed. Second chloroform (1-5 mL chloroform per 10 g silicone-glucose mixture) was added to decrease the viscosity of the mixture allowing it to flow more easily. Thereafter, 100 µL of the compounds were filled at the bottom of each cavity of a 2.2 mL polypropylene microtiter plate (HJ Bioanalytik, Mönchengladbach, Germany) using a multipette (Eppendorf, Wesseling-Berzdorf, Germany). The plate was stored at 50°C for 12 h to aid cross-linking. The final plate was then gamma-sterilized. The controlled-release fed-batch microtiter plates were produced at the Institute for Textile Chemistry and Macromolecular Chemistry (ITMC) of the RWTH Aachen University (Aachen, Germany).

Cultivation

For reproducible inoculation of the example precultures in this work, additional first cultures were performed to provide biomass. The first cultivations were conducted in Wilms-MOPS synthetic medium and Syn6-MES medium with 20 g/L glucose for *E. coli* and *H. polymorpha*, respectively. The following cultivation parameters were applied: 350 rpm shaking frequency (n), 50 mm shaking diameter (d_0), 10 mL filling volume (V_L) in 250 mL shake flasks. These first cultures were centrifuged and washed two times in 5 mL fresh medium and optical densities (OD) were measured. The OD values were used for the calculation of the required inoculation volume for each described experiment. All cultures in shake flasks were conducted in an in-house made Respiration Activity Monitoring System (RAMOS) for online-monitoring of oxygen transfer rates (OTR), as previously described by Anderlei et al. (Anderlei and Büchs 2001a; Anderlei et al. 2004a). A commercial version of this device is available from HiTec Zang GmbH (Herzogenrath, Germany) or Kühner AG (Birsfelden, Switzerland). The following cultivation parameters were applied: 350 rpm shaking frequency, 50 mm shaking diameter, 10 mL filling volume in 250 mL RAMOS flasks. The applied fed-batch mode in shake flasks was realized by using three controlled-release discs per flask. These controlled-release discs (denominated as 'FeedBeads') contain a silicone elastomer matrix in which glucose is embedded. They are available from Adolf Kühner AG, Birsfelden, Switzerland. In general, the discs with silicon elastomer did not adversely affect microbial growth (Jeude et al. 2006b).

For fed-batch precultivations, deepwell plates with immobilized silicon elastomer depots at the bottom of each well (FeedPlates) were used under the following conditions: 700 µL filling volume, 25 mm shaking diameter and 400 rpm shaking frequency. The microtiter plates were sealed with an airpore-sheet (nonwoven sealing foil, HJ Bioanalytik, Mönchengladbach, Germany) and cultivated under a humified aerated hood to minimize evaporation. The substrate released up to the time t can be described as follows:

$$\text{glucose}-\text{release} = 2 \cdot t^{0.69} \quad \text{[mg/disc]} \quad \text{for Feed Beads and} \quad (6\text{-}1)$$

$$\text{glucose}-\text{release} = 0.8 \cdot t^{0.72} \quad \text{[mg/well]} \quad \text{for FeedPlates.} \quad (6\text{-}2)$$

The glucose release kinetic of Equation 6-2 is shown in Fig. 6-1D. For subsequent fed-batch precultivations, the first cultures were centrifuged and resuspended in glucose-free medium to

prevent possible residues of glucose from the first cultures. All cultures were incubated at 37°C in shakers of type LS-W or ISF-4-W from Adolf Kühner AG (Birsfelden, Switzerland).

Analytical methods

Optical density of microtiter plate experiments was measured at 600 nm (OD_{600}) with the microtiter plate reader Powerwave X340 (Bio-Tek Instruments GmbH, Bad Friedrichstal, Germany) and for shake flask experiments a Uvikon 922 spectrophotometer (Kontron, Milano, Italy) was used. Samples were measured in the linear range of OD measurements after dilution in fresh medium. Sampling and OD measurement were each conducted twice or thrice. Samples from the fed-batch microtiter plates were withdrawn from different wells. Otherwise, the lower volume and the continuous release of substrate would have led to glucose concentrations that are higher than those in the unsampled wells.

6.3 Theoretical Background

A simple model for batch and fed-batch cultivations in microtiter plates was applied to demonstrate the concept of equalizing the growth in precultures. The variation of initial biomass concentrations (inocula), lag phase and specific growth rates was chosen to visualize growth and substrate kinetics of different precultures. These cultures may represent various clones of a clone library.

Modeling

A simple model for fed-batch cultivations in microtiter plates with standard bioreaction equations based on Monod kinetics was applied (Eq. 6-3 to 6-7).

$$\frac{dX}{dt} = (\mu - K_d) \cdot X \qquad [g/L/h] \qquad (6\text{-}3)$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \mu_{max} \cdot X + \text{Feedrate} \qquad [g/L/h] \qquad (6\text{-}4)$$

$$\mu = \frac{\mu_{max} \cdot S}{(S + K_S)} \cdot rel_{act} \qquad [1/h] \qquad (6\text{-}5)$$

$$\text{Feedrate} = \frac{0.8 \cdot 0.72 \cdot t^{(-0.28)}}{V} \qquad [g/L/h] \qquad (6\text{-}6)$$

$$rel_{act} = \frac{1}{1 + e^{\left(2+4 \cdot \frac{t - t_{lag}}{t_{acc}}\right)}} \qquad [-] \qquad (6\text{-}7)$$

Eq. (6-6) was obtained by differentiating Eq. (6-2) and referring it to the utilized filling volume in a well. Eq. 6-7 is an equation developed by us to represent the lag and acceleration phase of a culture. The term rel_{act} (relative activity) in equation 6-5 and 6-7 accounts for the lag phase and acceleration phase and varies from 0 (start of cultivation) to 1 (cells adapted to the new medium). The advantage over existing equations is that the two parameters t_{lag} and t_{acc} can directly be interpreted as the time constant for the lag and acceleration phase, respectively. A decay term was introduced to simulate the decrease in biomass due to the lack of a carbon source and the accumulation of end products in the stationary phase of batch cultivations. As there is no general consensus for mathematical modeling of a decay term or death rate (Toal et al. 2000), a constant decay term was assumed in the model (Moser and Steiner 1975). Simulations were conducted with Modelmaker (Cherwell Scientific, Oxford, UK).

Typical values for precultivation parameters of *E. coli* were introduced into the model (K_S = 0.2 g/L, t_{acc} = 0.5 h, K_d = 0.01 1/h, Y_{XS} = 0.5 g/g, S_0 = 15 g/L for batch, S_0 = 0 g/L for fed-batch). All the other model parameters were selected according to the caption of Fig. 6-1. The volume of the precultivation was set at 700 µL as a typical value for small-scale cultivations in deepwell microtiter plates. Within the simulation time of 34 h, the initial glucose concentration (substrate S_0) in the batch model was comparable to the total amount of glucose fed in the fed-batch mode. The term *Feedrate* in equation 6-4 was omitted for the batch simulations.

Batch cultivation

After the lag phase, the different batch precultivations grow exponentially (Fig. 6-1A) until the substrate is exhausted (Fig. 6-1B). Then, the stationary phase begins and the decay of viable biomass becomes apparent. This applies for all four simulations which results in different growth kinetics and physiological states at any time in batch mode. Moreover, non-optimal conditions for inoculation of the main culture are realized in the stationary phase (see arrow in Fig. 6-1A), because the clones are nutrient depleted in the stationary phase for different times. When these cells are used as a preculture for screening experiments, differences in growth kinetics in the main cultivation are most probable (see chapter 1.2).

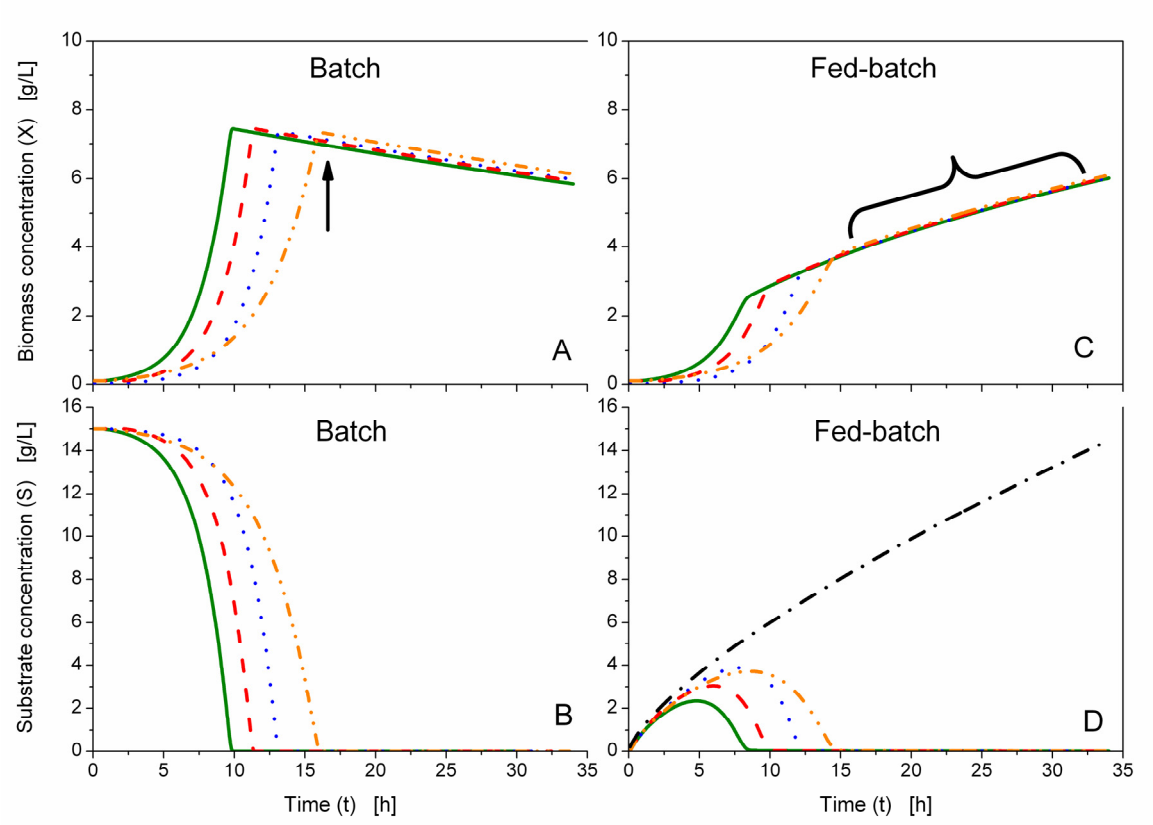

Fig. 6-1: Comparison of batch and fed-batch modes for cultivating precultures. Variations in lag phase, maximum specific growth rate or initial biomass concentration were simulated. (A) growth kinetics in batch mode; (B) substrate kinetics in batch mode; (C) growth kinetics in fed-batch mode; (D) substrate kinetics in fed-batch mode. (—) reference growth parameters (X_0 = 0.1 g/L; t_{lag} = 0.5 h; μ_{max} = 0.5 1/h); (---) decreased initial biomass concentration (X_0 = 0.02 g/L; t_{lag} = 0.5 h; μ_{max} = 0.5 1/h); (•••) increased lag time (X_0 = 0.1 g/L; t_{lag} = 2.0 h; μ_{max} = 0.5 1/h); (–••–) decreased μ_{max} (X_0 = 0.1 g/L; t_{lag} = 0.5 h; μ_{max} = 0.3 1/h); (–•–) total release of substrate from controlled-release system in fed-batch mode (calculated using Eq. 6-2). The arrow and the bracket indicate the time for inoculation of the main culture from batch and fed-batch preculture, respectively.

Fed-batch cultivation

The fed-batch mode allows predefined growth behavior of the preculture via feeding and, thus, offers the advantage of a more controlled process than the batch mode. In fed-batch simulations, shown in Fig. 6-1C and D, the addition of glucose to the applied medium was unnecessary, because of the immediate release of glucose from the release system. This is also apparent in Fig. 6-1D, which shows the total release of glucose throughout the cultivation. The substrate accumulates at the beginning of the fermentation when the cell concentration is still too low to consume the released glucose. After 5 to 10 h the biomass of the simulated precultures consume more glucose than provided by the controlled-release system and, consequently, the substrate concentration decreases (Fig. 6-1D). The preculture turns from a batch to a fed-batch phase after 8 to 15 h (Fig. 6-1C). In this phase the organisms are growing in a substrate-limited fashion and show a growth rate predefined by the feed rate of the

controlled-release system (Eq. 6-6). It is obvious that the fed-batch mode equalizes the different growth behavior of all precultures. The cells are constantly supplied with substrate, thereby resulting in constant growth and a defined metabolic state from 15 h onwards (Fig. 6-1C). In this way, the adverse effects of nutrient starvation or accumulation of overflow metabolites are minimized.

This system is self regulating concerning the consumption of glucose of each preculture. After the different bacterial clones switched over to glucose-limited growth, they all consume equal amounts of glucose and, hence, produce equal cell densities. The simulation demonstrates that fed-batch fermentations with defined feeding rates can equalize precultures that have different inocula, specific growth rates and lag phases. Moreover, the time for inoculation of main cultures is, in contrast to batch-precultures, no longer important. Even if the transfer of inocula is postponed relative to a fixed schedule, e.g. due to some practical reasons, the precultures do not suffer from carbon source depletion (see bracket in Fig. 6-1C) and preserve their metabolic activity.

6.4 Results and Discussion

RAMOS cultivations

Batch Mode

Batch precultures of the recombinant strain *E. coli* BL21 pRset eYFP-IL6 were cultivated as reference at three different initial optical densities (OD_{to}) from 0.1 to 0.5. They were inoculated from the same first culture in order to provide defined starting conditions. These represent a range commonly applied when inoculating precultures. The oxygen transfer rates are depicted in Fig. 6-2 against the fermentation time. The oxygen transfer rate (OTR) signal shows typically exponential growth and oxygen is limited only for a short period (plateau) upon attaining an OTR of approximately 60 mmol/L/h. When glucose is exhausted, the OTR decreases sharply (e.g. at 12 h for the culture with OD_{to} = 0.1). Due to overflow metabolism, acetate is formed during the exponential growth phase; its assimilation marks the second peak in the OTR curve of each culture. These phenomena have been described for this strain in chapter 3. The different inocula resulted in a time variation of approximately 4 h in growth.

The results demonstrate clearly the significant difference in growth kinetics of the microorganisms caused only by different initial biomass concentrations.

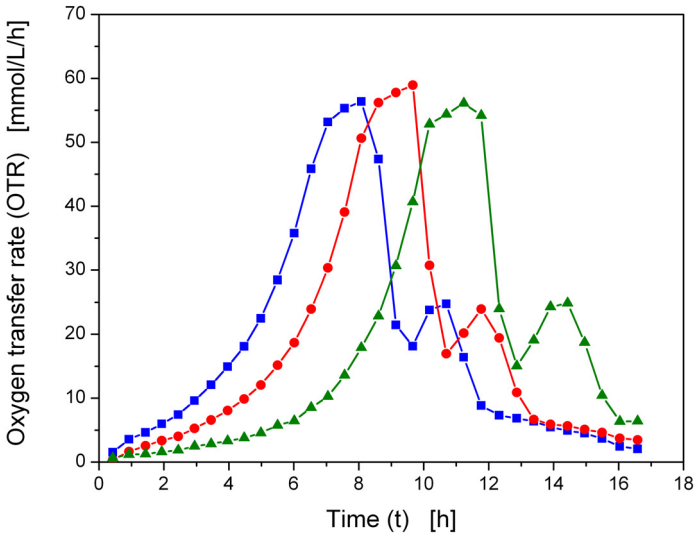

Fig. 6-2: Batch cultivation of *E. coli* BL21 pRset eYFP-IL6 in RAMOS flasks with various initial biomass concentrations: OD_{to} = 0.5 (■), OD_{to} = 0.3 (●), OD_{to} = 0.1 (▲). Wilms-MOPS medium with 15 g/L glucose, T = 37 °C, V_L = 10 mL, n = 350 rpm, d_0 = 50 mm.

Fed-batch mode

For fed-batch precultures in shake flasks, FeedBeads were applied, and the fermentation was monitored online with the RAMOS device (Fig. 6-3A).

The exponential growth of the batch phase in the beginning is followed by substrate limitation and a sharp OTR decrease. It is noteworthy that this decrease does not fall to zero but rather to a defined value of approximately 3 mmol/L/h. This value reflects the constant release of the substrate from the FeedBeads during the fed-batch phase. This growth pattern is obvious for all of the three differently inoculated precultures. The OTR peak of the batch phase increases for precultures with less inoculum (from 10 mmol/L/h for OD_{to} = 0.5 to 17 mmol/L/h for OD_{to} = 0.1; Fig. 6-3A). This increase is caused by the fact that more glucose accumulates in the flasks with lower inoculum before the fed-phase starts (see also Fig. 6-1D). Nevertheless, as soon as all precultures have reached the fed-batch mode (11 h), they all have consumed the same total amount of glucose. In fed-batch mode neither acetate formation nor oxygen limitation can be observed due to the lower initial glucose concentration and subsequent glucose-limited growth as compared with the batch mode (compare Fig. 6-3A and Fig. 6-2).

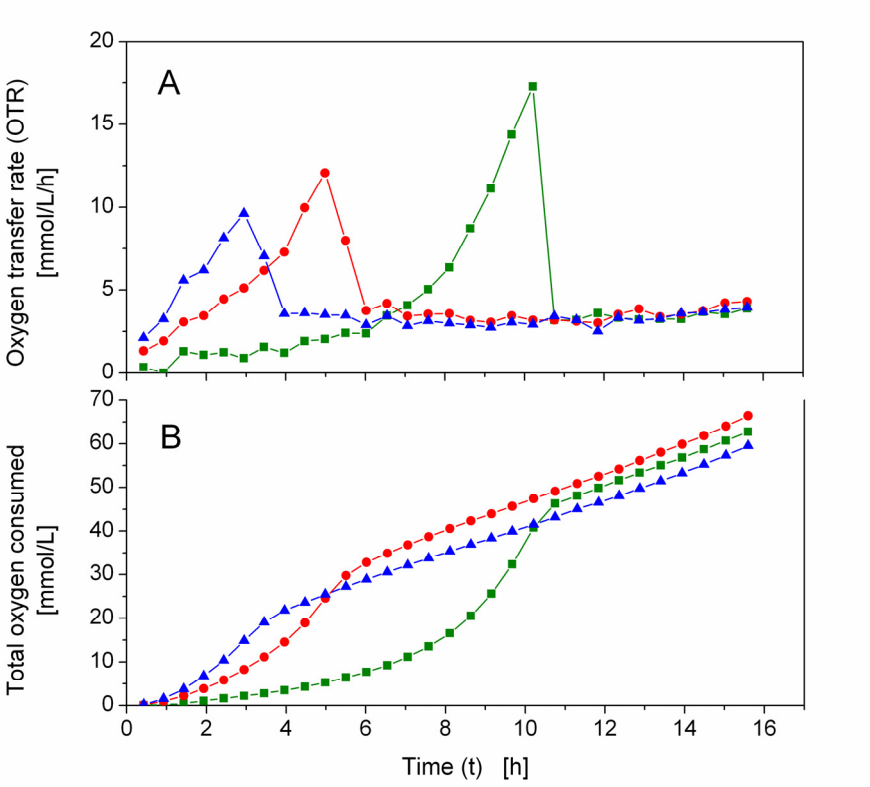

Fig. 6-3: Fed-batch precultivation of *E. coli* BL21 pRset eYFP-IL6 in RAMOS flasks with various initial biomass concentrations: OD_{to} = 0.5 (▲), OD_{to} = 0.3 (●), OD_{to} = 0.1 (■). (A) Oxygen transfer rate and (B) calculated total oxygen consumption. Wilms-MOPS medium with no additional glucose, 3 FeedBeads per flask, T = 37 °C, V_L = 10 mL, n = 350 rpm, d_0 = 50 mm.

All the precultures are found to be in a defined and similar metabolic state beginning at 11 h and are actively growing. At the end of the experiment, the OD of the precultures with an initial biomass of OD_{to} of 0.1, 0.3 and 0.5 were 4.4, 4.5 and 4.2, respectively. This demonstrates equalized growth.

Figure 6-3B shows the total oxygen consumed by the cells at any given time obtained by the integration of the OTR. By assuming a constant yield coefficient of biomass to oxygen during fermentation, the total oxygen consumption reflects the increase in biomass and is in good agreement with the simulated data (Fig. 6-1C). Variations in the total oxygen consumption during the fed-batch phase (Fig. 6-3B) result from errors in integrating curves with only few data points.

Fed-batch in microtiter plates

The newly developed fed-batch system for microtiter plates was used. Two microorganisms were tested to equalize the various precultures. Initial optical densities of 0.05, 0.1 and 0.3 were applied to simulate variable growth kinetics. The different precultures were inoculated from the same first culture. For *E. coli,* varying the inoculum from the highest to the lowest initial biomass concentration yielded a ca. 10 h delay in growth (Fig. 6-4). In fed-batch mode the precultures turned one after another from an exponential growth to a controlled nearly linear increase in biomass concentration. After approximately 15 h, all precultures were equalized and attained the same biomass concentration of OD 6.5 at the end of the experiment. As previously mentioned, this effect was caused by the same total amount of glucose being released per well.

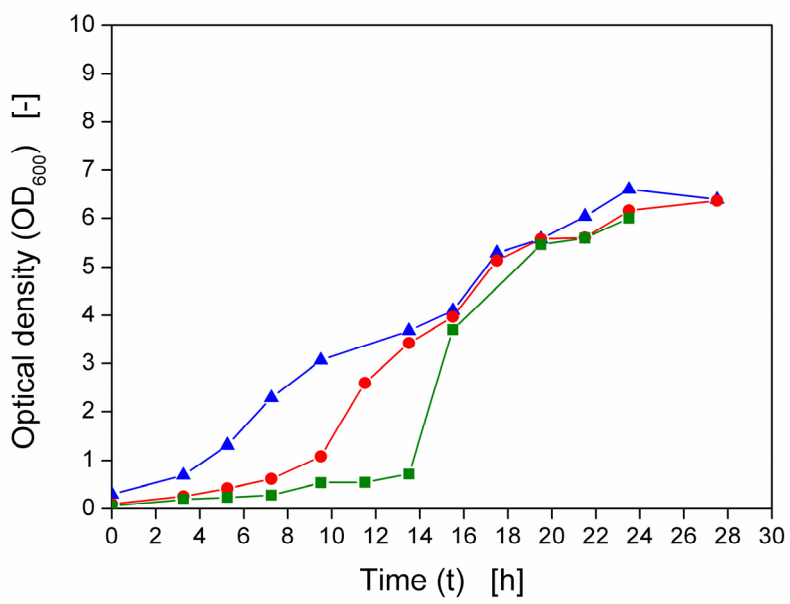

Fig. 6-4: Fed-batch precultivation of *E. coli* BL21 pRset eYFP-IL6 in a fed-batch deepwell plate with various initial biomass concentrations: OD_{to} = 0.5 (▲), OD_{to} = 0.3 (●), OD_{to} = 0.1 (■). Wilms-MOPS medium with no additional glucose, T = 37 °C, V_L = 700 µL, n = 400 rpm, d_0 = 25 mm.

The same behavior as for *E. coli* can be observed for *Hansenula polymorpha* precultures. Here, a 10 h delay in growth is observed for precultures with high and low inoculum concentration (Fig. 6-5). The applied fed-batch mode enables equalization of all precultures after 24 h. At 50 h the different cultures still have the same biomass concentration and are actively growing.

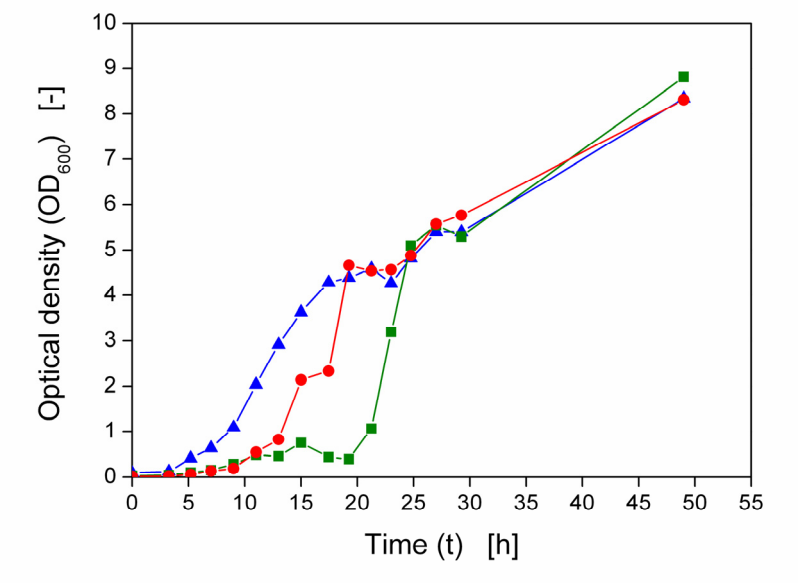

Fig. 6-5: Fed-batch precultivation of *Hansenula polymorpha* in a fed-batch deepwell plate with various initial biomass concentrations: OD_{to} = 0.5 (▲), OD_{to} = 0.3 (●), OD_{to} = 0.1 (■). Wilms-MOPS medium with no additional glucose, T = 37 °C, V_L = 700 µL, n = 400 rpm, d_0 = 25 mm.

6.5 Conclusions

A new technique for growth equalization in high-throughput precultivations by applying fed-batch mode was simulated and verified experimentally. Growth of differently inoculated precultures in shake flasks and microtiter plates could be equalized and, therefore, the feasibility of this concept could be demonstrated. The concept worked for prokaryotic and eukaryotic microorganisms. Consequently, this technique seems to be of general applicability.

The inherent advantages of this method are that it is easy to use as it requires no additional equipment for fed-batch precultivations on a small-scale. Furthermore, there is no need for permanent and laborious offline-monitoring of precultures to determine the right time of transfer to a main cultivation. Moreover, the exact time for inoculation of main cultures is, in contrast to batch precultures, no longer important. The system is self-regulating; the cells are continuously supplied with substrate and are in a defined metabolic state. This represents the strategy for achieving uniform conditions in the growth of different clones as recommended by Studier (Studier 2005), with the exception that the microorganisms are not in the stationary growth phase. Possible oxygen limitations and adverse effects of the batch mode can also be avoided in the fed-batch mode.

This technique might be especially useful for microorganisms exhibiting decreasing viability in the stationary phase and in which synchronous growth of distinct precultures is very important. Furthermore, precultures in fed-batch mode microtiter plates can generate more relevant data in screening processes (Jeude et al. 2006), because the starting conditions for all strains under study are equal. A possible disadvantage of the introduced equalization technique may be the expression of toxic products in hosts which are de-repressed by low glucose concentrations during the fed-batch phase. Further investigations have to be performed to prove this method with different clone libraries and the impact of equalized precultures on product formation (e.g. recombinant proteins or amino acids) in subsequent main cultivations.

7. Conclusions and Outlook

7.1 Conclusions

Screening experiments are of utmost importance for developing biotechnological processes. As the cultivation parameters of the screening for a rational selection of production microorganisms should match the parameters in the production process, the scale-down of the cultivation parameters from the large-scale to the small-scale is absolutely necessary. The optimization of the screening conditions involves different challenges and problems as described in chapter 1.2. Therefore, different facets of screening processes were analyzed and solutions for their optimization were investigated within this thesis.

To enhance the online information obtained during cultivation experiments in shake flasks, the RAMOS device for measuring respiration activities in shake flaks and a fiber optical, online pH-measurement technique were successfully combined as described in chapter 3. With this combination the pH-values during cultivations in RAMOS flasks and normal shake flasks can be compared more efficiently. Moreover, the pH effects on the OTR during growth of microorganisms, e.g. inhibited growth due to too low pH values, can easily be identified with this measuring setup. The combination of the online OTR and pH-measurement gives a lot of detailed information about the cultivation and, therefore, is a powerful tool for monitoring shake flask experiments for screening processes as well as for process development. Furthermore, this technique allows a more efficient development of controlled-release systems for controlling pH-values in shake flaks (see chapter 4).

One important difference between fermentations in various scales is the active pH-control in large-scale and in contrast, the high buffer concentrations used in shaken bioreactors for controlling the pH. A new polymer-based controlled-release system for controlling the pH-value also in shake flasks was presented in chapter 4. This controlled-release system consists of a polymer matrix in which sodium carbonate as pH-control reagent is encased. The

successful application of the controlled-release of sodium carbonate with defined release kinetics in cultivations of two different *E. coli* strains in mineral media with either glycerol or glucose as carbon sources was demonstrated. It was possible to substantially reduce the buffer concentrations in media for shake flask cultures, while the pH-values remain in the physiological range of microbial growth during the whole cultivation. This reduction in buffer concentration leads to reduced osmolarities in the medium. Furthermore, the applied conditions with less buffer concentrations enhance the comparison of small-scale and large-scale fermentation processes and thus enable a more reliable scale-up of experiments.

For screening cultures the growth behavior of the microorganisms has a great impact, for example, on the induction time of protein expression (see chapter 6). Especially different lag times of the microorganisms considerably affect the screening process. Therefore, the effect of the initial pH-value on the lag phase of four *E. coli* strains in a buffered minimal medium was investigated in chapter 5. For three *E. coli* strains a lower initial pH-value resulted in a shorter lag phase, whereas one *E. coli* strain showed the opposite behavior. Therefore, the influence of the initial pH-value seems to be strain-dependent. In contrast to the beneficial shorter lag phase with decreasing initial pH-values of the three *E. coli* strains mentioned above, the increasing acidification of the respective media during the cultivation eventually hampered *E. coli* growth. This effect could be avoided by cultivating at a higher initial pH-value which, in turn, would lengthen the lag phase. Increasing the buffer concentration could be one solution to this dilemma, although a higher buffer concentration would cause a higher osmotic pressure with a potentially adverse impact on the growth of *E. coli*. Furthermore, for process development, the potential benefits from shorter lag phases could be exploited, if the reaction of the applied *E. coli* strain on initial pH-values is taken into account. For future large-scale cultivation a low initial pH-value could, for example, be applied to shorten the lag phase of the fermentation. After the lag phase the pH-value could be increased by adding alkalizing reagents, thus controlling the pH at optimal values for growth and product formation. In screening experiments this effect on the lag phase should always be considered, because the different lag phases lead to unequal growth in both the screening main culture and also the respective preculture, which then substantially influences the subsequent screening main culture (see chapter 6).

Besides the described differences in the initial pH-values, differences in the inoculation of precultures in shake flasks and microtiter plates have a tremendous effect on the microbial

growth and thus on rational design of screening processes. These effects may be caused, for example, by different initial biomass concentrations in the preculture or strains inoculated in different growth phases. Therefore, a new technique for growth equalization in high-throughput precultivations by applying fed-batch mode was introduced in chapter 6. The presented concept worked for the bacterium *E. coli* and the yeast *H. polymorpha*. The inherent advantage of this method is the easy handling of the applied polymer-based controlled-release system as it requires no additional equipment for high-throughput fed-batch precultivations on a small-scale. Another advantage is that no permanent and laborious offline-monitoring is needed to determine the right time for inoculation of a main cultivation. Additionally, the exact time to inoculate the main cultivation, in contrast to batch precultures, is no longer important. This technique might be especially useful for microorganisms exhibiting decreasing viability in the stationary phase and in which synchronous growth of distinct precultures is very important. This newly developed method for precultivating microorganisms in fed-batch mode microtiter plates can generate more relevant data in screening processes (Jeude et al. 2006), because the starting conditions for all investigated strains are equal. Therefore, the optimal operational mode for performing screening processes for developing fed-batch production processes should be the fed-batch cultivation mode, as stated by Stöckmann et al. (2009) and Scheidle et al. (2010) with fed-batch precultures as demonstrated in this work.

The polymer-based controlled-release fed-batch microtiter plate presented in chapter 6 is an ideal tool for high-throughput screenings. In contrast to other mini bioreactor systems, such as the BioLector, the cultivation in this fed-batch method does not contain the online measurement of different parameters. This system does not mandatory need these additional devices for a controlled growth of the microorganisms, because it is self regulating. Furthermore, the easy-to-use controlled-release microtiter plates can be handled like normal microtiter plates in high-throughput applications with dozens of parallel experiments. Especially the combination with an automatic liquid handling system, the usage of the plates would immensely increase the throughput of the controlled-release fed-batch system.

To improve screenings, the developed and applied polymer-based controlled-release systems have been proven to be very useful. With these systems it is possible to scale-down essential cultivation parameters from large-scale to small-scale screening, such as fed-batch mode and low buffer concentrations established with the pH-control. The experiments described in this

work suggest, that it is preferable to transfer the complete screening - preculture and main culture - to fed-batch operational mode in order to select the most suitable microorganisms for a subsequent fed-batch production process.

7.2 Outlook

For further optimizing screening parameters it would be very interesting to use the methods demonstrated here, to perform an exemplary process development. In these experiments the whole process concerning further problems in scale-down can be analyzed and additional differences between the various scales can be identified. Thereby, controlled-release fed-batch precultures and fed-batch main cultures for the screening of a model clone library could be used. Then, a scale-up into laboratory-scale could be done to confirm the proposed better screening results, while comparing them with results from a conventional screening. Here, the influence of complex and mineral media and the osmotic pressure on the selection of microorganisms can be investigated. Furthermore, the results of chapter 5 demonstrated the strain dependent influence of the initial pH-value in the media on the lag phase of *E. coli* cultures. It would be very interesting to investigate this influence on the lag phases of different further microorganisms such as other bacteria and yeasts. Moreover, the combination of these methods with 'omics-technologies', such as transcriptomics, proteomics and metabolomics, would give further information about the influence of the cultivation parameters on the regulation and the metabolic flux of the microorganisms. With this information production microorganisms and process parameters can be adapted to an optimal screening and production process.

In cooperation with the Institute for Textile Chemistry and Macromolecular Chemistry (ITMC) of the RWTH Aachen University, new controlled-release systems will be developed for different applications in microbial cultivations. For example, the polymer-based controlled-release system for controlling the pH-value in shaken bioreactors (presented in chapter 4) will be further developed to enable a self regulating system. This new system will use, for instance, pH-sensitive polymers to establish a release of pH-control reagents dependent on the actual pH-value in the surrounding medium. The pH-sensitive polymers will, for example, increase the release of the pH-control reagent with decreasing pH-values below 7 and will decrease the release at pH-values of 7 and above. It will then be possible to

use this system under a lot of different cultivation conditions, because the release kinetics is not predefined. Moreover, with such a pH-sensitive controlled-release system, it will be possible to cultivate microorganisms in small-scale shaken bioreactors without any additional buffer, thereby guaranteeing constant growth conditions. A transfer of this controlled-release system into microtiter plates will than give an easy-to-use pH-control in high-throughput cultivations. A future combination of the controlled-release systems for fed-batch cultivation and pH-control would, thus, enable the user to perform screenings under the same conditions as applied in large-scale processes. Furthermore, polymer-based controlled-release systems with different release kinetics and different release substrates will be developed. For example, a system for feeding ammonium chloride into plant cell cultures, with release kinetics over 7 days is currently tested. Furthermore, the usage of an automatic liquid handling robot would increase the high-throughput potential of the controlled-release microtiter plates for the screening of hundreds or thousands of different clones under fed-batch and low osmolarity conditions.

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